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The Component Acids of the Perinephric and Interscapular Fats of a Rabbit

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(*Received* 22 *February* 1951)

The component fatty acids and glycerides of the fats of comparatively few species of land animals have been studied in detail. Information so far available relates mostly to the body fats of the domestic animals, ox, sheep and pigs, and to cow and buffalo milk fats. The data available for the component acids of a greater but still limited number of animal fats, however, indicate that a progressive change in the direction of simplification tends to occur as one proceeds from the lower to the higher land animals.

The fats of only two species of rodents have so far been examined in detail, the component acids being recorded respectively for those of male and female white rats (Longenecker, 1939), and of healthy guinea pigs (*Cavia cutleri*) (Baldwin & Longenecker, 1944) fed on a low-fat content diet. The values obtained are quoted in Table 3, together with those of Vickery (recorded by Hilditch, 1947) for the fat of the wild rabbit and with the values obtained in the present investigation for the component acids of the perinephric and interscapular fats of an experimental rabbit.

Lewkowitsch (1922) has recorded iodine values of 101 and 64·4 for the fats from wild and tame rabbits (*Lepus cuniculus*), respectively, noting that the fat from the wild animal dries to a nearly solid varnish in 7 days, whereas that from the tame rabbit exhibits no drying properties. The fat from a wild rabbit, studied by Vickery and reported by Hilditch (1947), had a saponification equivalent of 285·2, and iodine value 124. The component acids included myristic 5%, palmitic 23%, and stearic 4% with 68% of unsaturated acids, the latter having an iodine value of 189·3. The unsaturated acids were not completely identified, but it was shown that linoleic acid was present to the extent of 35%, whilst linolenic acid was present to the extent of about 20%. This fat, therefore, only resembled that of the rat and the guinea pig in so far that the saturated acids comprise about 30% of the mixed fatty acids, but differed completely from them in the proportions of the unsaturated acids present.

The perinephric and interscapular fats from a healthy Chinchilla rabbit, which had been receiving a daily low-fat diet consisting of 12 oz. (340 g.) green stuff and 4 oz. (110 g.) rat cake (the latter containing 5–6% fat) and water three times a week, have now been investigated.

EXPERIMENTAL

The fatty tissues were dissected out immediately after slaughter and extracted with light petroleum (b.p. 40–60°) and gave fats of the following characteristics:

	Saponification equivalent	Iodine value
Perinephric fat	283·1	72·3
Interscapular fat	283·7	66·3

Determination of the component fatty acids

The mixed acids obtained after hydrolysis of the perinephric fat were resolved by low-temperature crystallization into three fractions consisting of mainly polyethenoid, mainly monoethenoid and mainly saturated acids, respectively, whereas the acids from the interscapular fat were resolved into two fractions mainly unsaturated and mainly saturated, respectively. Each fraction was methylated and fractionated through an electrically heated and packed column, the saponification equivalent and iodine value of each ester fraction being determined. Spectrophotometric examination (Hilditch, Morton & Riley, 1945) of the acids

Table 1. *Low-temperature crystallization of rabbit fat, mixed fatty acids*

	Perinephric			Interscapular		
	Weight			Weight		
	(g.)	(%)	Iodine value	(g.)	(%)	Iodine value
A. Insoluble in ether at −35°	18·58	37·6	7·6	14·31	34·0	5·3
B. Soluble in ether at −35°	15·11	30·5	83·2	27·74	66·0	102·5
C. Soluble in ether at −60°	15·80	31·9	141·1	—	—	—

Table 2. *Component acids of perinephric and interscapular rabbit fats*

	Perinephric fat					Interscapular fat			
	Acid groups			Total fatty acids		Acid groups		Total fatty acids	
Acid	*A* (37·6 %*) (%†)	*B* (30·5 %*) (%†)	*C* (31·9 %*) (%†)	excluding unsaponifiable (%, w/w)	(% by mol.)	*A* (34·0 %*) (%†)	*B* (66·0 %*) (%†)	excluding unsaponifiable (%, w/w)	(% by mol.)
Lauric	—	—	1·1	0·4	0·5	—	3·7	2·4	3·2
Myristic	5·8	8·0	2·8	5·5	6·5	7·3	2·0	3·8	4·5
Palmitic	72·1	8·2	2·3	30·5	3·19	75·8	4·7	29·0	30·2
Stearic	12·0	1·6	—	5·0	4·7	11·9	—	4·0	3·8
Dodecenoic	—	—	—	—	—	—	0·1	0·1	0·1
Tetradecenoic	—	—	—	—	—	—	2·2	1·4	1·7
Hexadecenoic	—	4·1	12·9	5·4	5·7	—	9·9	6·6	6·9
Hexadecadienoic	—	—	0·5	0·2	0·2	—	0·2	0·1	0·1
Hexadecatrienoic	—	—	1·0	0·3	0·3	—	0·3	0·2	0·2
Oleic	9·9	70·1	20·9	31·9	30·4	3·8	53·4	36·7	34·6
Linoleic	—	6·7	44·7	16·3	15·6	1·1	17·2	11·8	11·2
Linolenic	—	1·3	8·4	3·1	3·1	—	3·0	2·0	1·9
Unsaturated C_{20-22}	—	—	4·5	1·4	1·2	—	2·8	1·9	1·6
Unsaponifiable	0·2	—	0·9	—	—	0·1	0·5	—	—

* Group as % (w/w) of total fat. † Component acids as % (w/w) of group.

Table 3. *Component acids of rodent fats (% by mol.)*

	Rats (Longenecker, 1939)		Guinea pig (Baldwin & Longenecker, 1944)	Experimental rabbit (Present work)		Wild rabbit (Hilditch, 1947)
Acid	Male	Female		Perinephric	Interscapular	
Lauric	—	—	1·5	0·5	3·2	—
Myristic	1·6	1·8	6·4	6·5	4·5	5
Palmitic	21·6	24·4	20·6	31·9	30·2	25
Stearic	3·6	3·6	5·5	4·7	3·8	4
Arachidic	2·0	0·8	—	—	—	—
Dodecenoic	—	—	—	—	0·1	—
Tetradecenoic	—	0·3	1·0	—	1·7	12
Hexadecenoic	4·1	4·8	2·2	5·7	6·9	
Hexadecadienoic	—	—	—	0·2	0·1	
Hexadecatrienoic	—	—	—	0·3	0·2	
Oleic	51·9	44·3	34·9	30·4	34·6	
Linoleic	13·0	18·6	18·3	15·6	11·2	34
Linolenic	—	—	1·2	3·1	1·9	20
Unsaturated C_{20-22}	2·2	1·4	8·4	1·2	1·6	—

recovered from selected ester fractions enabled the amounts of hexadecadienoic and octadeca-di- and octadeca-tri-enoic acids to be estimated, respectively. The octadeca-di- and octadeca-tri-enoic acids were further characterized and found to consist for the most part, if not entirely, of ordinary seed-fat linoleic and linolenic acids, since tetrabromostearic acid, m.p. 114°, and hexabromostearic acid, m.p. 180°, were readily obtained from suitable ester fractions.

DISCUSSION

It is seen that there is no major difference in the compositions of the perinephric and interscapular fats of the rabbit, the saturated acid contents in particular being very similar. The interscapular fat is, however, somewhat more saturated than the perinephric fat, this being due to an increase in the amount of oleic acid (about 4 %) at the expense of a corresponding decrease in its linoleic acid content. This is of considerable interest, since it is known that the perinephric fats are more saturated than fats located at other sites in the body for other animals so far investigated. The hexadecenoic acid content of both fats is of the order of 5–7 %, this value being of the same order as that which obtains in the body fats of rats and guinea pigs (Table 3). Hexadeca-di- and hexadeca-tri-enoic acids in small amounts were found to accompany the hexadecenoic acid in both perinephric and interscapular fats.

Judged from the composition of their fats the rodents appear to occupy an interesting transitional position in the scale of biological evolution. Thus the palmitic acid content is of the order of 20–30 % of the amount characteristic of the higher land animals, whereas the hexadecenoic acid content is considerably higher than that which obtains in these animals, indicating that rodent fats are inter-

mediate in type between those of the higher land animals and those of the reptiles and amphibia so far reported, in which the palmitic acid content is somewhat reduced, being of the order of 10–20%, hexadecenoic acid being present in these fats to the extent of 5–15%.

Table 3 records the component acids of the rodents so far investigated. It is seen that the fats of the tame rabbit are on the whole similar to those of the other (tame) rodents. The palmitic acid content of the rabbit fats, however, is somewhat higher than that of the rat and guinea pig fats. The unsaturated C_{18}-acid content resembles that of the guinea pig rather than that of the rats, the guinea pig, however, elaborating a considerable amount of polyethenoid C_{20-22} acids, the amount being of the order of that present in the fats of Amphibia and reptiles. The saturated acids of the wild rabbit fat are likewise of the same order as those found in the fats of the tame rodents, but there is a marked difference in the proportions of the unsaturated acids present.

It appears, therefore, that the fats of the tame rodents fed on a suitable low-fat diet are generally similar, but judged from the only detailed data available there is reason to believe that the type of fat elaborated by the tame rodents is not typical of that of the wild varieties of the same species.

SUMMARY

1. The composition of the perinephric and interscapular fats of an experimental rabbit has been investigated.

2. The proportions of the component acids of both fats were very similar, the perinephric fat being somewhat the more unsaturated.

3. Both fats were broadly similar to those of other tame rodents, but differ appreciably from that recorded for a wild rabbit.

We wish to thank Prof. T. P. Hilditch, F.R.S., for his sustained interest in this investigation, and Prof. R. A. Morton, F.R.S., for placing the rabbit at our disposal.

REFERENCES

Baldwin, A. R. & Longenecker, H. E. (1944). *Arch. Biochem.* **5**, 147.

Hilditch, T. P. (1947). *The Chemical Constitution of Natural Fats*, 2nd ed. pp. 84, 298. London: Chapman and Hall.

Hilditch, T. P., Morton, R. A. & Riley, J. P. (1945). *Analyst*, **70**, 68.

Lewkowitsch, J. (1922). *Chemical Technology and Analysis of Oils, Fats and Waxes*, vol. **2**, pp. 680, 691. London: Macmillan and Co.

Longenecker, H. E. (1939). *J. biol. Chem.* **128**, 645.

Studies in the Biochemistry of Micro-organisms

84. THE COLOURING MATTERS OF *PENICILLIUM NALGIOVENSIS* LAXA. PART 1. NALGIOVENSIN AND NALGIOLAXIN. ISOLATION, DERIVATIVES AND PARTIAL STRUCTURES

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(*Received* 20 *February* 1951)

Penicillium nalgiovensis Laxa was first described by Laxa (1932), who isolated it during an examination of the microflora of Ellischauer cheese, a form of Camembert cheese peculiar to the Nalžovy region of Southern Bohemia—hence the specific name. The production of this type of cheese was started about 1880 in an attempt to reproduce the flavour and aroma of Camembert cheese. During the ripening process, the surface of the cheese is covered with white, velvet-like mycelium which gradually becomes greenish-grey in colour. The surface eventually becomes flecked with reddish patches from which cultures of *P. nalgiovensis* can be isolated. According to Laxa this mould is one of the principal species associated with the ripening process. The species is included by Raper & Thom (1949) in the *P. canescens* series of the Asymmetrica-divaricata. Growth of the species on cheese and in laboratory culture, particularly on media containing proteins, is associated with the production of considerable amounts of red pigment. The species has not been reported from any other source than Ellischauer cheese.

It was noted in this laboratory that the mycelium of *P. nalgiovensis*, grown on Raulin-Thom solution, gave strong red colours when moist portions of it were immersed in cold concentrated sulphuric acid or in aqueous sodium hydroxide, and pale to light red colours when immersed in aqueous sodium carbonate. On exhaustive extraction of the dried mycelium with light petroleum an orange solid

separated in the boiling solvent. Purification of this solid by crystallization from chloroform led to the isolation of a pure, hitherto undescribed, colouring matter for which the name *nalgiovensin* is proposed. Only a portion of the total colouring matters present in the mycelium is extracted with light petroleum. Subsequent extraction with ether or, preferably, with chloroform, yields further amounts of nalgiovensin and a second hitherto undescribed colouring matter for which the name *nalgiolaxin* is proposed. Nalgiovensin constitutes about 1·0% of the dry weight of the mycelium of *P. nalgiovensis*, and nalgiolaxin about 0·18%.

Nalgiovensin crystallizes from chloroform in long orange needles, and from glacial acetic acid in glistening orange plates, m.p. 199–200°, without decomposition. It has the molecular formula

$$C_{18}H_{16}O_6, \text{ i.e. } C_{17}H_{13}O_5.OCH_3,$$

contains one methyl group attached to carbon, one methoxyl group, and is dextrorotatory in chloroform solution $[\alpha]_{5461}^{20°}+48·1°$; $[\alpha]_{5790}^{20°}+39·7°$ (*c*, 0·1786). Nalgiovensin does not dissolve in aqueous 0·5N-sodium bicarbonate or in N-sodium carbonate, but dissolves readily in N-sodium hydroxide to give a deep-red solution with a slight bluish cast. It dissolves in cold concentrated sulphuric acid to an intense red solution which appears somewhat orange in shade by transmitted light. Its solution in glacial acetic acid is yellow in colour and does not show fluorescence in daylight.

The following functional derivatives of nalgiovensin were prepared. (*a*) *Triacetylnalgiovensin*, $C_{24}H_{22}O_9$, i.e. $C_{17}H_{10}O_2(OCH_3)(OCOCH_3)_3$, by heating with acetic anhydride and anhydrous sodium acetate. It forms fine yellow needles, m.p. 147–148°, and is laevorotatory in chloroform solution. On alkaline hydrolysis of this acetate nalgiovensin is regenerated. (*b*) *Nalgiovensin dimethyl ether*, $C_{20}H_{20}O_6$, i.e. $C_{17}H_{11}O_3(OCH_3)_3$, by heating with dimethyl sulphate and anhydrous potassium carbonate in acetone solution. It forms yellow needles, m.p. 187–188°, which are insoluble in aqueous N-sodium hydroxide, indicating the absence of a nuclear hydroxyl group, but dissolve in cold concentrated sulphuric acid to give an intense red solution with a slight bluish cast. It is dextrorotatory in chloroform solution. (*c*) *Monoacetylnalgiovensin dimethyl ether*, $C_{22}H_{22}O_7$, i.e.

$$C_{17}H_{10}O_2(OCH_3)_3(OCOCH_3),$$

by heating nalgiovensin dimethyl ether with acetic anhydride and anhydrous sodium acetate. It forms thin yellow-green rods, m.p. 152·5–153°, which are dextrorotatory in chloroform solution. (*d*) *Nalgiovensin trimethyl ether*, $C_{21}H_{22}O_6$, i.e.

$$C_{17}H_{10}O_2(OCH_3)_4,$$

by heating nalgiovensin dimethyl ether with methyl iodide and dry silver oxide in dry benzene solution. It forms fine yellowish-green needles, m.p. 177–178°, which are insoluble in aqueous N-sodium hydroxide, but dissolve in cold concentrated sulphuric acid to yield a solution indistinguishable in colour from that given by the dimethyl ether. It is dextrorotatory in chloroform solution. Methylation of nalgiovensin with dimethyl sulphate and 10% aqueous potassium hydroxide resulted only in the formation of the dimethyl ether.

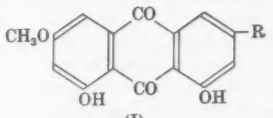
It appeared probable from the colour reactions of nalgiovensin and of its derivatives, and from its solubility in alkalis that this colouring matter is a polyhydroxyanthraquinone. This opinion was confirmed and the general structure of nalgiovensin was indicated by the fact that when a chloroform solution of it was chromatographed on a magnesium carbonate column a single red band was formed. This band became bluish-red and was considerably diffused when the column was washed with absolute methanol. Almost identical colours are given by physcion, 4:5-dihydroxy-7-methoxy-2-methylanthraquinone, structure (I) R = CH_3, under the same conditions.

The main features of the molecular constitution of nalgiovensin became clear as the result of oxidation experiments on nalgiovensin and its functional derivatives. Emodic acid trimethyl ether (4:5:7-trimethoxyanthraquinone-2-carboxylic acid) was obtained by the oxidation of nalgiovensin trimethyl ether with chromic acid in glacial acetic acid solution and also by the oxidation of nalgiovensin dimethyl ether with alkaline iodine in aqueous dioxan solution, in which oxidation no iodoform could be detected. On mild oxidation of nalgiovensin with chromic acid in glacial acetic acid solution at 60° there was obtained a small yield of 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid (I), R = COOH, together with a much larger yield of an at present unidentified oxidation product having the molecular formula $C_{18}H_{14}O_6$. This substance which contains the same number of carbon and oxygen atoms as, but has two hydrogen atoms less than, nalgiovensin is, unlike nalgiovensin, optically inactive. We believe it to have structure (I) in which R = C_3H_5O containing a CO group in place of CHOH.

On oxidation of triacetylnalgiovensin with chromic acid in a mixture of glacial acetic acid and acetic anhydride there is formed 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (diacetylemodic acid 7-methyl ether), i.e. the diacetate of (I), R = COOH, m.p. 214–215°. The structure of this oxidation product was proved by direct comparison of it with the oxidation product of diacetylphyscion which we prepared by a similar oxidation process. The two oxidation products proved to be identical. Their identity was confirmed by alkaline hydrolysis

of each of them. Both gave rise to 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid (I), R = COOH, m.p. and mixed m.p. 322–323°. Chromic acid oxidation of carbon side chains attached to the anthraquinone nucleus, with the formation of the corresponding anthraquinone carboxylic acids, has been used frequently in the investigation of the molecular structure of naturally occurring polyhydroxyanthraquinones, e.g. triacetylemodin gives triacetylemodic acid, $CH_3 \rightarrow COOH$ (Fischer & Gross, 1911; Eder & Hauser, 1925; Anslow, Breen & Raistrick, 1940); diacetylphyscion gives 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (diacetylemodic acid 7-methyl ether), $CH_3 \rightarrow COOH$ (Eder & Hauser, 1925); triacetylhelminthosporin gives triacetylhelminthosporic acid, $CH_3 \rightarrow COOH$ (Charles, Raistrick, Robinson & Todd, 1933); tetra-acetyl-ω-hydroxyemodin gives triacetylemodic acid, $CH_2OAc \rightarrow COOH$ (Anslow *et al.* 1940). If we accept that in the parallel oxidation of triacetylnalgiovensin to 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid the reaction follows a similar course, certain important conclusions follow.

(1) That nalgiovensin has the same nuclear structure (I) as is present in physcion, i.e.



(I)

where $R = CH_3$ in physcion and $R = C_3H_7O$ in nalgiovensin.

(2) That the C_3H_7O side chain in nalgiovensin must contain a hydroxyl group to account for the formation of triacetylnalgiovensin, nalgiovensin trimethyl ether and monoacetylnalgiovensin dimethyl ether, and may therefore be written C_3H_6OH. This conclusion is supported by the facts that in the oxidation of triacetylnalgiovensin one acetyl group is lost, just as is the case in the oxidation of tetra-acetyl-ω-hydroxyemodin to triacetylemodic acid, and that although nalgiovensin contains three hydroxyl groups which can be acetylated with acetic anhydride and sodium acetate, only two of them—almost certainly the nuclear hydroxyl groups—can be methylated with dimethyl sulphate and potassium carbonate, methylation of the third hydroxyl group—almost certainly that in the side chain—requiring the use of methyl iodide and silver oxide, a process which is known to methylate aliphatic hydroxyl groups, e.g. in sugars (Purdie & Irvine, 1901, 1903).

(3) That the C_3H_7O side chain must contain an asymmetric carbon atom to account for the optical activity of nalgiovensin and its functional derivatives—an unusual property of naturally occurring anthraquinones except of those conjugated ones occurring as glycosides.

(4) That the C_3H_7O side chain must therefore have one of the following configurations:

(*a*) $-CHOH.CH_2.CH_3$;
(*b*) $-CH_2.CHOH.CH_3$;
(*c*) $-CH(CH_3).CH_2OH$.

The evidence at present available favours either (*a*) or (*b*), since oxidation of side chain (*a*) to $-CO.CH_2.CH_3$ or (*b*) to $-CH_2.CO.CH_3$ would lead to loss of optical activity, whereas oxidation of (*c*) would lead to $-CH(CH_3).CHO$ which still contains an asymmetric carbon atom, and, as has been mentioned previously (see p. 564), mild oxidation of nalgiovensin with chromic acid in acetic acid solution at 60° gives a good yield of $C_{18}H_{14}O_6$ which is optically inactive.

The main conclusions reached above were confirmed by the observation that, when nalgiovensin was reduced with hydriodic acid and red phosphorus in glacial acetic acid solution and the resulting anthranol was oxidized with chromic acid in acetic acid solution, there was formed a new anthraquinone, $C_{17}H_{14}O_5$. This substance has the following properties. (*a*) It no longer contains a methoxyl group and, unlike nalgiovensin, is readily soluble in N-sodium carbonate as would be expected when the CH_3O group in position 7 is replaced by a free hydroxyl group. (*b*) It is optically inactive, whereas nalgiovensin is dextrorotatory. This is readily explained by postulating that the C_3H_6OH side chain in nalgiovensin is reduced to C_3H_7 in a manner analogous to that of the reduction, by the same reagents, of aloe-emodin, 4:5-dihydroxy-2-hydroxymethylanthraquinone, to chrysophanic acid, 4:5-dihydroxy-2-methylanthraquinone (Oesterle, 1911), and of ω-hydroxyemodin, 4:5:7-trihydroxy-2-hydroxymethylanthraquinone, to emodin, 4:5:7-trihydroxy-2-methylanthraquinone (Anslow *et al.* 1940). (*c*) It gives colour reactions which are indistinguishable from those given by emodin. (*d*) It forms a triacetyl derivative. We therefore formulate this reduction product as 4:5:7-*trihydroxy*-2-*propylanthraquinone* in which the nature of the propyl group, whether *n*-propyl from the side chains $-CHOH.CH_2.CH_3$ or $-CH_2.CHOH.CH_3$, or *iso*-propyl from the side chain $-CH(CH_3).CH_2OH$, is left undetermined for the present. We hope to obtain conclusive evidence on the nature of this propyl group and also on the exact structure of the C_3H_7O side chain in nalgiovensin by means of synthetic experiments which are in progress at present and will be reported later.

The second colouring matter, nalgiolaxin, present in the mycelium of *P. nalgiovensis* has the molecular formula $C_{18}H_{15}O_6Cl$ and is, we believe, the first example of a derivative of anthraquinone containing

chlorine which has been recorded from any natural source. The presence of chlorine in nalgiolaxin would appear at first sight to be an anomaly since Raulin-Thom solution, on which the mould was grown (for composition see p. 567), does not contain any intentionally added source of chlorine. We are, however, informed by the manufacturers (Corn Products Co. Ltd.) of the glucose (dextrosol monohydrate) used in the medium that the chloride content, calculated as sodium chloride, of this grade of glucose lies between 0·035 and 0·04%, and is therefore approximately four to five times the amount required for the production of nalgiolaxin in the yields which were actually obtained. Further, as will be shown later, nalgiolaxin is a monochloro derivative of nalgiovensin, and, since chloroform and hydrochloric acid are used in the preferred method of extraction and purification of nalgiolaxin, it became necessary to prove that nalgiolaxin is not an artifact. This was done (for details see p. 572) by avoiding the use of any reagents containing chlorine in the isolation and purification process. The colouring matter so isolated proved to be identical with nalgiolaxin obtained by the preferred isolation procedure.

Nalgiolaxin crystallizes from ethanol in small yellow plates and needles, m.p. 248–248·5°, without decomposition. Like nalgiovensin it contains one methyl group attached to carbon, one methoxyl group and is dextrorotatory in chloroform solution: $[\alpha]_{5790}^{22°} + 40·3°$. Its colour reactions are, in general, very similar to those of nalgiovensin and physcion, except that its solution in cold concentrated sulphuric acid is much bluer in tone and its red chromatogram on magnesium carbonate is also a shade bluer. Its solution in glacial acetic acid is yellow in colour and does not fluoresce in daylight.

Two functional derivatives of nalgiolaxin were prepared by the same methods as were used for the preparation of the corresponding derivatives of nalgiovensin. *Triacetylnalgiolaxin*, $C_{24}H_{21}O_9Cl$, i.e. $C_{17}H_9O_2Cl(OCH_3)(O.COCH_3)_3$, lemon-yellow plates, m.p. 186·5–187°, is, like triacetylnalgiovensin, laevorotatory in chloroform solution. *Nalgiolaxin dimethyl ether*, $C_{20}H_{19}O_6Cl$, i.e. $C_{17}H_{10}O_3Cl(OCH_3)_3$, yellow needles, m.p. 206–207°, is, like nalgiovensin dimethyl ether, dextrorotatory in chloroform solution.

The close structural relationship between nalgiovensin and nalgiolaxin and the fact that they both possess the same anthraquinone skeleton structure was proved by reducing nalgiolaxin with hydriodic acid and red phosphorus and oxidizing the resulting anthranol with chromic acid. There was thus obtained the same optically inactive 4:5:7-trihydroxy-2-propylanthraquinone as was formed on similar treatment of nalgiovensin. In both cases demethylation and reduction of the hydroxyl group in the side chain took place, and, in addition, in the case of nalgiolaxin the chlorine in this substance was replaced by hydrogen. The removal of halogen (bromine) from the anthraquinone nucleus by means of hydriodic acid was used by Jacobson & Adams (1924) in their synthesis of emodin, so that our findings provide presumptive evidence that the chlorine atom in nalgiolaxin is present in the anthraquinone nucleus and not in the 3-carbon side chain.

This important point was proved by oxidizing triacetylnalgiolaxin with chromic acid in acetic acid-acetic anhydride solution. There was thus obtained a compound, $C_{20}H_{13}O_9Cl$, i.e.

$$C_{15}H_4O_4Cl(OCH_3)(O.COCH_3)_2.$$

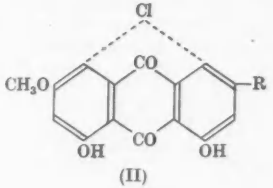
Triacetylnalgiovensin, on similar treatment, gives a compound, $C_{20}H_{14}O_9$, i.e.

$$C_{15}H_5O_4(OCH_3)(O.COCH_3)_2,$$

which, as previously stated, was identified as 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid, i.e. the diacetate of (I), R = COOH. The two oxidation products have almost identical colour reactions, only differing in the fact that, while both of them are readily soluble in cold 0·5N-sodium bicarbonate, indicating the presence in each of a carboxyl group, the nalgiolaxin oxidation product gives a much redder solution, as might be expected from the presence in it of a nuclear chlorine atom. Hence we feel justified in postulating that the nalgiolaxin oxidation product is a nuclear monochloro derivative of 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid, i.e. the diacetate of (II), R = COOH.

We have at present no conclusive evidence of the exact position of the chlorine atom in the anthraquinone nucleus of nalgiolaxin. It must, however, be in one of the four available positions, i.e. 1, 3, 6 or 8. Further, if it were replaced by a hydroxyl group this group would be in one of the same four positions, and, if this hydroxyl group were in either position 1 or 8, but not in positions 3 or 6, the resulting polyhydroxyanthraquinone would be a derivative of quinizarin, 1:4-dihydroxyanthraquinone, and all known quinizarin derivatives give solutions in acetic acid which fluoresce in daylight (Raistrick, Robinson & Todd, 1934). Replacement of a nuclear halogen by hydroxyl was described by Ullmann & Schmidt (1919), who converted 1-chloro-4-hydroxy-2-methylanthraquinone into 1:4-dihydroxy-2-methylanthraquinone, i.e. 2-methylquinizarin, by heating with concentrated sulphuric acid and boric acid at 150–160°. The same reaction was also used for the replacement of a nuclear anthraquinone halogen atom by hydroxyl by Hayashi (1927) in the synthesis of 8-methylquinizarin, and by Anslow & Raistrick (1941) in the synthesis of catenarin, 1:4:5:7-tetrahydroxy-2-methylanthraquinone. When nalgiolaxin was heated with concentrated sulphuric acid and boric acid the initially red solution changed colour

and became permanganate purple at 150–160°. A red colouring matter, at present not characterized, was recovered from the reaction mixture. Its solution in glacial acetic acid was orange-yellow in colour with a fine green fluorescence. It readily dissolved in aqueous N-sodium hydroxide and N-sodium carbonate to give blue-violet solutions. With concentrated sulphuric acid it gave a red-violet solution having a slight red fluorescence. These reactions are in marked contrast to the colours and non-fluorescent solutions given by nalgiolaxin in the same reagents and are very similar to those given by catenarin, 1:4:5:7-tetrahydroxy-2-methylanthraquinone, except that a solution of catenarin in cold concentrated sulphuric acid is much more blue-violet in colour. We believe that these observations, although at present purely qualitative, justify the tentative suggestion that the chlorine atom in nalgiolaxin is in either position 1 or 8, and that nalgiolaxin is therefore 1- or 8-monochloronalgiovensin (II), in which $R = C_3H_7O$ as in nalgiovensin.



(II)

EXPERIMENTAL

All melting points are uncorrected.

C, H, and Cl estimations were carried out by Drs Weiler and Strauss, Oxford, other estimations by one of us (J.Z.).

A 4 dm. tube was used for determination of optical rotations.

History of culture

The strain of *P. nalgiovensis* Laxa used throughout this work was received in July, 1948, from Dr K. B. Raper of the Northern Regional Research Laboratory (N.R.R.L.), United States Department of Agriculture, Peoria, Illinois, U.S.A. This culture, N.R.R.L. 911 (Thom's No. 5337.2), was originally described by Laxa in 1932 and was received from him by Dr C. Thom in April, 1933. A full description of the strain is given by Raper & Thom (1949, p. 319).

Cultural conditions

P. nalgiovensis does not sporulate heavily on beer-wort agar slopes. For this reason it was found desirable, for inoculation purposes, to sporulate the organism on sterile, moistened wheat bran. The bran spore inoculum was prepared as follows. Bran (5 g.) contained in a conical flask (250 ml.) was thoroughly moistened with tap water (4·5 ml.). The flask was plugged with cotton wool and autoclaved at 121° for 1 hr. After cooling, the bran was inoculated with *P. nalgiovensis* and incubated in the dark at 24° for 10–14 days. The mould grew uniformly throughout the bran and sporulated fairly well. A spore suspension was prepared by shaking the sporulated bran culture with 150 ml. of sterile dilute agar solution (0·125%) and pipetting off the suspended mould spores. The residual bran was re-treated with a second portion of agar solution (100 ml.) which was combined with the first suspension. A sterile, perforated sleeve fitted on the end of the pipette prevented the inclusion of any bran particles. The mould spore concentration of the combined suspensions was approximately 1,000,000/ml.

Batches of a hundred 1 l. conical flasks were prepared, each containing 350 ml. of Raulin-Thom solution: i.e. glucose, 75·0 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; $(NH_4)_2SO_4$, 0·25 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $FeSO_4.7H_2O$, 0·07 g.; $ZnSO_4.7H_2O$, 0·07g.; distilled water, 1500 ml. After sterilization each flask was inoculated with 4 ml. of the spore suspension described above. The inoculated flasks were incubated at 24° in the dark. A thin white vegetative mat was visible by the 2nd day and was well formed by the 3rd day. On the 5th day the mycelium was light creamy-green in colour with a light-yellow reverse, and had become pale yellow-green with a yellow-brown reverse by the 9th day. When harvested, at the end of 28 days, the mycelium was heavily folded and quite brittle. It was dull greyish-green in colour with a dark-brown reverse.

In order to determine the optimum time for harvesting the cultures fifteen flasks were harvested after 7, 14, 21, 28 and 35 days' incubation. Determinations were made of pH (colorimetrically) and residual glucose (by polarimeter) in the culture filtrates, and weight of mycelium, dried to constant weight in a desiccator over conc. H_2SO_4, and of crude nalgiovensin isolated from the dried mycelium as described below. The results obtained are shown in Table 1.

Isolation and purification of nalgiovensin

Batches of a hundred flasks were harvested at the end of 28 days' incubation. The brownish culture fluid was separated by decantation from the mould mycelium and was discarded. The mycelium was washed with distilled water, pressed as dry as possible in a tincture press and dried in a vacuum oven at 45–50°. The dried mycelium was ground to

Table 1. *Cultural characteristics of* Penicillium nalgiovensis *Laxa*

Incubation period (days)	No. of flasks	pH	Glucose by polarimeter (%)	Mycelium wt. (g.)	Crude nalgiovensin wt. (g.)
0	2	4·1	5·34	—	—
7	15	3·6	4·18	23·0	0·02
14	15	3·4	3·00	36·0	0·07
21	15	3·4	2·66	40·5	0·11
28	15	3·4	2·53	42·0	0·12
35	15	3·2	2·39	42·7	0·12

a fine powder in a coffee mill and was then distributed equally between five large Soxhlet thimbles and extracted in five Soxhlets with light petroleum (b.p. 40–60°) for 60–100 hr. During the extraction crude nalgiovensin separated from the boiling solution as an orange solid which is practically insoluble in boiling light petroleum. Approximately 45% of the total weight of nalgiovensin present in the mould mycelium is extracted under these conditions. Complete extraction of the pigment was accomplished only after subsequent extended extraction with $CHCl_3$ (see p. 571). After cooling, the crude nalgiovensin was separated by filtration and the orange-yellow light petroleum mother liquors, on removal of the solvent, left a small quantity of crude fat containing only traces of pigment. From a total of 989 flasks 2966 g. of dried mycelium were obtained yielding, on extraction with light petroleum, 8·97 g. of crude fat and 13·17 g. of crude nalgiovensin.

The crude nalgiovensin obtained by the above procedure melts at approximately 190° and was easily purified by successive crystallizations from $CHCl_3$, yielding about 50% of its weight of pure nalgiovensin, m.p. 199–200°. Pure nalgiovensin could not readily be obtained by fractional crystallization of the dried $CHCl_3$ mother liquors, nor could these be satisfactorily purified by chromatography on $MgCO_3$ columns. They were, however, easily purified by acetylation and fractional crystallization from dilute acetic acid of the crude triacetylnalgiovensin. The pure triacetylnalgiovensin thus obtained, m.p. 147–148°, was then hydrolysed with aqueous NaOH to give pure nalgiovensin (see below).

General properties of nalgiovensin. Derivatives

Nalgiovensin crystallizes from $CHCl_3$ as beautiful, long, flat, orange needles, and from glacial acetic acid as glistening orange plates, m.p. 199–200°, without decomposition. (Found, on sample sublimed in a high vacuum at 160–170°: C, 66·1, 65·95; H, 4·9, 4·9; CH_3O, 9·3, 9·4; *C*-CH_3, 5·0; N, S, Cl, nil. $C_{18}H_{16}O_6$ requires C, 65·9; H, 4·9; $1CH_3O$, 9·45; $1C$-CH_3, 4·6%.) It is dextrorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{20°}+48·1°$; $[\alpha]_{5790}^{20°}+39·7°$ (*c*, 0·1786). It readily sublimes in a high vacuum at 160–170° without decomposition. It readily dissolves in warm $CHCl_3$, glacial acetic acid and acetone, less readily in ethanol, and is only slightly soluble in light petroleum, b.p. 40–60°. It does not dissolve in 0·5 N-$NaHCO_3$ or in N-Na_2CO_3, but dissolves readily in aqueous NaOH to a red solution with a slight bluish cast. It dissolves in cold conc. H_2SO_4 to a beautiful red solution which appears somewhat orange by transmitted light. Its ethanolic solution gives an orange-brown colour with ethanolic $FeCl_3$. Its solution in glacial acetic acid is yellow in colour and does not fluoresce in daylight. Its $CHCl_3$ solution gives a pure red band when chromatographed on columns of $MgCO_3$ (British Drug Houses Ltd., 'heavy'). The band becomes bluish-red and is considerably diffused when the column is washed with absolute methanol.

Triacetylnalgiovensin. Anhydrous sodium acetate (0·4 g.) was added to a solution of nalgiovensin (0·186 g.) in acetic anhydride (2 ml.), and the mixture was maintained at 100° for 2 hr. It was then cooled and poured into ice water. The yellow solid which separated (0·248 g.) was filtered, dried and crystallized from dilute ethanol to give fine, yellow needles of *triacetylnalgiovensin*, m.p. 147–148°. (Found, on sample dried in a high vacuum at 70° with no loss in wt.: C, 63·8, 63·65; H, 5·4, 5·1; CH_3O, 6·8, 6·8; CH_3CO, by hydrolysis, 27·6, 28·3. $C_{24}H_{22}O_9$ requires C, 63·4; H, 4·9; $1CH_3O$, 6·8; $3CH_3CO$, 28·4%.) Triacetylnalgiovensin is laevorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{20°}-8·9°$; $[\alpha]_{5790}^{20°}-8·3°$ (*c*, 0·6076).

Triacetylnalgiovensin (0·67 g.) was heated for 3 hr. with aqueous N-NaOH (45 ml.) on a boiling-water bath in an atmosphere of N_2. The acetate quickly dissolved to give an intense wine-red solution. N-H_2SO_4 (50 ml.) was added and the resulting orange precipitate was coagulated by heating for 0·5 hr. and was then filtered, washed and dried (0·49 g.). The orange solid was sublimed and then crystallized from $CHCl_3$, wt. 0·37 g., m.p. 199–200°. The colouring matter was still dextrorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{20°}+48·5°$; $[\alpha]_{5790}^{20°}+40·4°$ (*c*, 0·2192).

Nalgiovensin dimethyl ether. A mixture of nalgiovensin (0·54 g.), anhydrous K_2CO_3 (10·8 g.), redistilled dimethyl sulphate (5·4 ml.) and dry acetone (300 ml.) was boiled under reflux for 8 hr., during which period the colour of the solution changed from deep wine-purple to pure lemon-yellow. The hot solution was filtered and evaporated to 20 ml. *in vacuo*. Water (500 ml.) and 2 N-HCl (50 ml.) were added. The yellow solid which separated was filtered, washed and dried (0·53 g.). It was dissolved in $CHCl_3$ (250 ml.) and traces of partially methylated material were removed by shaking the $CHCl_3$ solution with 0·5 N-NaOH and then with water. On removal of the $CHCl_3$ there remained a yellow solid (0·51 g.) which was purified by sublimation in a high vacuum at 180° (0·49 g., m.p. 187–188°) followed by crystallization from aqueous ethanol. *Nalgiovensin dimethyl ether* was thus obtained as thin, branching yellow needles, m.p. 187–188°. (Found: C, 67·2, 67·2; H, 5·8, 6·0; CH_3O, 25·5, 25·5. $C_{20}H_{20}O_6$ requires C, 67·4; H, 5·7; $3CH_3O$, 26·1%.) Nalgiovensin dimethyl ether is dextrorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{20°}+35·9°$; $[\alpha]_{5790}^{20°}+28·8°$ (*c*, 0·4620). It is readily soluble in $CHCl_3$, acetone and ethanol. It is insoluble in N-NaOH, but dissolves in cold conc. H_2SO_4 to an intense red solution with a slight bluish cast.

Monoacetylnalgiovensin dimethyl ether. Nalgiovensin dimethyl ether (0·13 g.) was acetylated in the usual manner with anhydrous sodium acetate (0·26 g.) and acetic anhydride (2 ml.). The yellow solid (0·16 g.) which separated on dilution of the acetylation mixture with water was crystallized from ethanol (7 ml.). *Monoacetylnalgiovensin dimethyl ether* was thus obtained as thin yellow-green rods (0·09 g.), m.p. 152·5–153°. (Found: C, 66·0, 66·1; H, 5·65, 5·6; CH_3O, 23·4. $C_{22}H_{22}O_7$ requires C, 66·3; H, 5·6; $3CH_3O$, 23·4%.) It is dextrorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{22°}+4·8°$; $[\alpha]_{5790}^{22°}+3·8°$ (*c*, 0·2512). Its solution in cold conc. H_2SO_4 is almost indistinguishable in colour from that given by nalgiovensin dimethyl ether.

Nalgiovensin trimethyl ether. Nalgiovensin dimethyl ether (0·30 g.) was dissolved in dry benzene (100 ml.) and methyl iodide (20 ml.). Dry Ag_2O (5 g.) was then added in portions at hourly intervals (2 g. at 0 hr. and 1 g. at the end of the first, second and third hour) and the mixture was heated under reflux for a total of 8 hr. The solvents were removed *in vacuo* and the resulting solid was extracted with warm benzene (5 × 20 ml.). The filtered, yellow benzene solution was evaporated *in vacuo* leaving a crystalline yellow solid (0·31 g., m.p. 176·5–177°) which was purified by crystallization from ethanol. *Nalgiovensin trimethyl ether* was thus obtained as fine, yellow-green needles (0·26 g.), m.p. 177–178°. (Found, on sample sublimed in a high vacuum at 170°: C, 67·9, 67·8; H, 6·1, 6·1; CH_3O, 33·1. $C_{21}H_{22}O_6$ requires C, 68·1; H, 6·0; $4CH_3O$, 33·5%.) It is dextrorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{21°}+6·7°$; $[\alpha]_{5790}^{21°}+5·8°$ (*c*, 0·4531). Its solution

in cold conc. H_2SO_4 is almost indistinguishable in colour from that given by nalgiovensin dimethyl ether.

Attempts to prepare nalgiovensin trimethyl ether by other recognized methods failed. Methylation of nalgiovensin with a large excess of dimethyl sulphate and 10% (w/v) aqueous KOH at 45–50° or at 100° yielded nalgiovensin dimethyl ether. Attempts to methylate nalgiovensin dimethyl ether further, either in methanol solution with dimethyl sulphate and 10% aqueous KOH or in acetone solution with ethereal diazomethane, yielded only unchanged nalgiovensin dimethyl ether.

OXIDATION EXPERIMENTS WITH NALGIOVENSIN AND ITS DERIVATIVES

(*a*) *With alkaline hydrogen peroxide*

H_2O_2 (100 vol., 5 ml.) was added drop by drop to a solution of nalgiovensin (0·1 g.) in N-NaOH (7·5 ml.), and the whole was maintained in an atmosphere of N_2. The wine-red solution was then slowly heated to 60°, without any evidence of a reaction occurring, and maintained at this temperature for 0·5 hr. The starting material was recovered unchanged by acidification with 2N-HCl (6 ml.) and subsequent sublimation of the recovered orange solid (m.p. and mixed m.p. 199–200°).

(*b*) *Mild* CrO_3 *oxidation of nalgiovensin*

A solution of CrO_3 (0·50 g.) in water (20 ml.) and glacial acetic acid (20 ml.) was quickly added with constant shaking to a solution of nalgiovensin (0·50 g.) in glacial acetic acid (200 ml.), the whole being maintained at 60°. The resulting brown solution was held at 60° for 0·5 hr. and was then poured into ice water (2 l.). The orange-yellow solid which separated was extracted from the solution with ether (3 × 1 l.). The golden-yellow ether solution was washed twice with water and was then extracted with 0·5N-$NaHCO_3$ (4 × 250 ml.). The reddish-orange $NaHCO_3$ extracts were washed twice with fresh ether, acidified with HCl and then re-extracted with $CHCl_3$ (3 × 50 ml.). The $CHCl_3$ solution was washed with water and evaporated to dryness, giving a red-orange solid (0·01 g.) which was crystallized from glacial acetic acid (8 ml.). 4:5-Dihydroxy-7-methoxyanthraquinone-2-carboxylic acid was thus obtained as orange needles, m.p. 320–321° (decomp.) and was identified by comparison with an authentic specimen (see § *c*) (m.p. 322–323° decomp., mixed m.p. 321–322° decomp.). The two specimens also gave indistinguishable colour reactions.

The neutral ether solution remaining after extraction with $NaHCO_3$ was washed with water and evaporated to dryness. The resulting orange solid (0·38 g.), m.p. 190·5–191·5°, was crystallized three times from ethanol giving beautiful, large, orange needles, m.p. 199–200°, mixed m.p. with nalgiovensin (m.p. 199–200°) being 178–185°. (Found: on sample sublimed in a high vacuum at 170–175°: C, 65·6; H, 4·3; CH_3O, 9·5, 9·2. $C_{18}H_{14}O_6$ requires C, 66·3; H, 4·3; 1CH_3O, 9·5%.) This *oxidation product*, which is optically inactive in $CHCl_3$ solution (*c*, 0·4556), gives colour reactions with conc. H_2SO_4 and N-NaOH which are identical with those given by nalgiovensin (see p. 568).

(*c*) CrO_3 *oxidation of triacetylnalgiovensin*

Triacetylnalgiovensin (1·13 g.) was dissolved at 55–60° in a mixture of glacial acetic acid (23 ml.) and acetic anhydride (23 ml.). The temperature was maintained at 55–60°, while a solution of CrO_3 (2·3 g.) in water (1·8 ml.) and glacial acetic acid (23 ml.) was gradually added with constant shaking over a period of 0·5 hr. The bath temperature was then raised to 65–70° and maintained at this level for 3 hr. The deep-green solution was poured into warm water (800 ml.) and left in the refrigerator overnight, when a yellow crystalline solid separated. The whole was extracted with $CHCl_3$ (3 × 150 ml.). The yellow $CHCl_3$ solution was first washed with water and was then extracted with 0·5N-$NaHCO_3$ (4 × 100 ml.) yielding (i) a $CHCl_3$ solution of neutral oxidation products, and (ii) an aqueous $NaHCO_3$ solution of acidic oxidation products.

(i) *Treatment of* '*neutral*' $CHCl_3$ *solution.* On removal of the $CHCl_3$ there remained a yellow oil (0·96 g.), which later solidified, of which 0·83 g. was successively re-oxidized three times yielding 0·47 g. of neutral fraction after the second oxidation, 0·31 g. after the third and 0·23 g. after the fourth oxidation. These neutral fractions consist of a mixture of neutral oxidation intermediates. In a second experiment the crude neutral fraction (0·18 g., m.p. 155·5–156·5°) obtained from the oxidation of 0·26 g. of triacetylnalgiovensin was repeatedly crystallized from aqueous ethanol and then from absolute ethanol finally giving beautiful, long, yellow needles (0·11 g.), m.p. 165–166°. (Found: C, 61·3, 60·95; H, 4·35, 4·6; CH_3O, 6·9, 6·7. $C_{24}H_{20}O_{10}$ requires C, 61·5; H, 4·3; 1CH_3O, 6·6. $C_{24}H_{22}O_{10}$ requires C, 61·3; H, 4·5; 1CH_3O, 6·6%.) The *oxidation product*, which appears to be optically inactive in $CHCl_3$ (*c*, 0·152), dissolves in cold conc. H_2SO_4 to a red solution indistinguishable in colour from that given by nalgiovensin with the same reagent.

(ii) *Treatment of* $NaHCO_3$ *solutions.* The four $NaHCO_3$ extracts were quickly and separately acidified with 2N-HCl and extracted with $CHCl_3$. On removal of the $CHCl_3$ from the four extracts there remained 0·09 + 0·20 + 0·06 + 0·03 g. = 0·38 g. of yellow solid. This was fractionally crystallized from ethanol and then from 50% aqueous acetic acid from which there separated first a small amount of a monoacetate and then finally a diacetate in long, thin, yellow needles, m.p. 214–215°, the overall yield of which was about 50% of the crude acid oxidation product. The diacetate was identified as 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (diacetylemodic acid-7-methyl ether). (Found: C, 60·2, 60·3; H, 3·85, 3·8; CH_3O, 7·7. Calc. for $C_{20}H_{14}O_9$: C, 60·3; H, 3·5; 1CH_3O, 7·8%.) It did not depress the m.p. (214–215°) of an authentic specimen prepared by oxidation of diacetylphyscion (see p. 570) and previously described by Eder & Hauser (1925). Both specimens gave the following reactions: 0·5N-$NaHCO_3$, yellow solution; N-Na_2CO_3, or N-NaOH, red solution with a slight bluish cast; conc. H_2SO_4, intense reddish-orange solution.

The identity of the oxidation diacetate was confirmed by hydrolysis to the parent anthraquinone. The diacetate (0·07 g.) was heated with aqueous 0·5N-NaOH (35 ml.) on a boiling-water bath for 2 hr. in an atmosphere of N_2. The intense wine-red solution was acidified with 2N-HCl (12 ml.) and heated for 0·5 hr. to coagulate the gelatinous orange precipitate which was filtered, washed and dried (0·045 g.). It was crystallized from glacial acetic acid as orange needles or as heavy reddish needle clusters, m.p. 322–323° (decomp.) alone or in admixture with 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid prepared from diacetylphyscion (see p. 570). Both specimens were readily soluble in cold 0·5N-$NaHCO_3$ to a reddish-orange solution and both gave reactions with N-Na_2CO_3, N-NaOH and cold conc. H_2SO_4 which were indistinguishable from those given by the diacetyl compound (see previous paragraph). The acid is

sparingly soluble in $CHCl_3$, ethanol and glacial acetic acid from which solvent it crystallizes with acetic acid of crystallization which is extremely difficult to remove. (Found, on sample dried in a high vacuum at 85° with no loss in wt.: C, 57·3, 57·2; H, 4·0, 3·75. $C_{16}H_{10}O_7 + C_2H_4O_2$, i.e. $C_{18}H_{14}O_9$, requires C, 57·8; H, 3·8. On sample sublimed in a high vacuum at 230–235°, m.p. 322–323° (decomp.): C, 61·4; H, 3·6. Calc. for $C_{16}H_{10}O_7$: C, 61·15; H, 3·2%.)

Preparation of 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid from physcion. A sample of the lichen *Xanthoria parietina* L. Fr. was collected from limestone walls at Oranmore, near Galway, Eire. The dried lichen (56·8 g.) was extracted with cold $CHCl_3$ (6 × 400 ml.). The dark solid (0·62 g.) remaining on removal of the solvent was purified by sublimation in a high vacuum at 160° (0·40 g.) and crystallization from glacial acetic acid (14 ml.). Physcion (4:5-dihydroxy-7-methoxy-2-methylanthraquinone) was thus obtained as flat, orange needles (0·31 g.), m.p. 206–207°, not depressed on admixture with an authentic specimen (m.p. 206–207°). The lichen physcion gives colours with cold conc. H_2SO_4 and N-NaOH which are indistinguishable from those given by nalgiovensin.

Physcion (0·20 g.) was acetylated with anhydrous sodium acetate (0·4 g.) and acetic anhydride (2 ml.) in the usual manner. Diacetylphyscion (0·26 g.), m.p. 185–187°, was obtained crystalline when the reaction mixture was poured into water. This acetate (0·26 g.), without any further purification, was dissolved in a mixture of glacial acetic acid (5·2 ml.) and acetic anhydride (5·2 ml.) at 55–60°, and oxidized at 65–70° for 3 hr. with CrO_3 (0·52 g.) dissolved in water (0·41 ml.) and glacial acetic acid (5·2 ml.). Oxidation of the methyl group in physcion to COOH was complete in one oxidation (cf. oxidation of triacetylnalgiovensin). The crude oxidation product was purified exactly as described in § *c*, ii, p. 569, and 4:5-diacetoxy-7-methoxyanthraquinone-2-carboxylic acid (0·10 g.) was thus obtained as long, thin, yellow needles, m.p. 214–215°. Eder & Hauser (1925) give the m.p. of this acid as 214–215°. This acetylated acid (0·09 g.) was hydrolysed with 0·5 N-NaOH (40 ml.) in N_2 (see p. 569). The resulting 4:5-dihydroxy-7-methoxyanthraquinone-2-carboxylic acid was obtained as heavy reddish needle clusters (0·05 g.), m.p. 322–323° (decomp.), by crystallization from glacial acetic acid. Eder & Hauser (1925) give the m.p. of this acid as 300°. Their preparation was crystallized from pyridine, the product containing pyridine of crystallization. The pyridine was removed by heating at 120°, the acid so obtained melting at 298° raised to 300° by sublimation *in vacuo* at an unspecified temperature. Our preparation contained acetic acid of crystallization, and when sublimed in a high vacuum at 250–260° the sublimate melted at 304–305°. Part of the same specimen sublimed at 230–235° gave a sublimate melting at 322–323°.

(*d*) *Alkaline iodine oxidation of nalgiovensin dimethyl ether*

The method used was essentially that described by Fuson & Bull (1934). 10% aqueous NaOH (1 ml.) was added to a solution of nalgiovensin dimethyl ether (0·1 g.) in dioxan (10 ml.). The iodine reagent (KI, 10 g.; I_2, 5 g.; water, 40 ml.) was added dropwise with constant shaking. There did not appear to be any decolorization and an excess of the reagent was added (7 ml.). The dark solution was heated and held at 60° for 1 hr. The excess I_2 colour was then discharged by the dropwise addition of 10% aqueous NaOH (approx. 3 ml.) and the yellow solution was diluted with water to 125 ml. After holding in the refrigerator overnight, the yellow solid which separated was filtered and dried (0·04 g.). It did not contain iodoform, and since it was shown to be a K salt it was decomposed by refluxing for 0·75 hr. in 50% aqueous acetic acid (30 ml.). On cooling, flat yellow needles (0·02 g.) separated, m.p. 268–269° (decomp.), alone or in admixture with authentic emodic acid trimethyl ether, m.p. 268–269° (decomp.). (Found: C, 60·3; H, 4·4. $C_{18}H_{14}O_7 . H_2O$ requires C, 60·0; H, 4·5%.) Emodic acid trimethyl ether crystallizes with 1 mol. water of crystallization.

The alkaline oxidation filtrate from the above K salt was extracted with $CHCl_3$ yielding 0·03 g. of unchanged nalgiovensin dimethyl ether. The $CHCl_3$-extracted alkaline solution was acidified with HCl and was aerated for a short time at 60° to eliminate the last traces of $CHCl_3$ and some of the liberated I_2. The yellow solid which separated on cooling was filtered, washed with water, dried (wt. 0·03 g.) and crystallized from ethanol (15 ml.) yielding 0·015 g. of emodic acid trimethyl ether in fine yellow needles, m.p. 267–268° (decomp.). (Found: C, 60·05; H, 4·5. $C_{18}H_{14}O_7 . H_2O$ requires C, 60·0; H, 4·5%.) In a repeat oxidation of 2 min. duration no iodoform was observed, 70% of the starting material was recovered unchanged and about 30% of emodic acid trimethyl ether was isolated.

Authentic emodic acid trimethyl ether was prepared by methylation of authentic emodic acid with dimethyl sulphate and aqueous KOH essentially as described by Eder & Hauser (1925). The crude methylation product was purified by crystallization from ethanol. It forms thin yellow needles, m.p. 268–269° (decomp.). Eder & Hauser (1925) give the m.p. as 270° (decomp.). The oxidation acid and authentic emodic acid trimethyl ether gave the following indistinguishable colour reactions: with cold conc. H_2SO_4 an intense red solution with a slight bluish cast; readily soluble in 0·5 N-$NaHCO_3$ and N-Na_2CO_3 to a yellow solution unchanged on standing; readily soluble in aqueous N-NaOH and 10% NaOH giving a yellow solution which becomes reddish on standing for 1 hr.; insoluble in 10% KOH, slightly soluble in 5% KOH, readily soluble in 1% KOH.

(*e*) CrO_3 *oxidation of nalgiovensin trimethyl ether*

Nalgiovensin trimethyl ether (0·23 g.) dissolved in glacial acetic acid (4·6 ml.) and acetic anhydride (4·6 ml.) was oxidized exactly as described for triacetylnalgiovensin (see § *c*) with CrO_3 (0·46 g.) in water (0·37 ml.) and glacial acetic acid (4·6 ml.). The oxidation appeared to proceed more quickly than that of triacetylnalgiovensin. The acid fraction obtained (0·02 g.), on crystallization from ethanol (3 ml.), yielded thin yellow needles, m.p. 267–268° (decomp.), not depressed on admixture with authentic emodic acid trimethyl ether. It also gave the same colour reactions (see § *d*).

REDUCTION OF NALGIOVENSIN

A mixture of nalgiovensin (0·50 g.), glacial acetic acid (10 ml.), red phosphorus (0·50 g.) and HI (2 ml. sp.gr. 1·7) was refluxed for 5 hr. The mixture was cooled and poured into water (500 ml.). Traces of I_2 were destroyed by the addition of a slight excess of aqueous $NaHSO_3$, and the suspended solid was separated by filtration, washed, dried and extracted with warm $CHCl_3$. After removal of the solvent the resulting

yellow solid was reduced a second time with fresh quantities of reagents. The filtered $CHCl_3$ solution, after the second treatment, was evaporated to low bulk. The *anthranol* crystallized as microplates (0·43 g.), m.p. 185°.

A small sample (0·01 g.) was recrystallized from $CHCl_3$-light petroleum (b.p. 40–60°) giving pale-yellow plates, m.p. 188°. It gave colour reactions typical of an emodin-type anthranol. It dissolved in cold conc. H_2SO_4 to a yellow solution which slowly became dark green on standing or more rapidly on warming. It readily dissolved in N-NaOH to a yellow solution which, on shaking, passed through a series of colours giving finally a stable red solution with a slight bluish cast. It did not dissolve in cold N-Na_2CO_3 except on long standing.

The anthranol (0·42 g.), m.p. 185°, without further purification, was dissolved in glacial acetic acid (95 ml.). The solution was maintained at 60° and a solution of CrO_3 (0·42 g.) in water (6 ml.) and glacial acetic acid (6 ml.) was quickly added with constant shaking. The mixture was held at 60° for 0·5 hr. and was then poured into ice water (500 ml.). The brown solid which separated was filtered, washed and dried (0·43 g.). It was purified by crystallization first from $CHCl_3$ and then from benzene. 4:5:7-*Trihydroxy-2-propylanthraquinone* was thus obtained as long yellow needles appearing orange in bulk, m.p. 216·5–217°. (Found, on sample crystallized from benzene and dried in a high vacuum at 100°, loss in wt. 9·6%: C, 68·1; H, 4·7. $C_{17}H_{14}O_5$ requires C, 68·45; H, 4·7%.) It is optically inactive in $CHCl_3$ (*c*, 0·2512). It readily sublimes in a high vacuum at 170°. It gives the following colour reactions. It is insoluble in aqueous 0·5N-$NaHCO_3$; it readily dissolves in cold conc. H_2SO_4, N-Na_2CO_3 and N-NaOH giving in each case a red solution with a slight bluish cast, the solution colours in N-Na_2CO_3 and N-NaOH being a stronger blue than that in conc. H_2SO_4. Emodin (4:5:7-trihydroxy-2-methylanthraquinone) gives identical colours with these reagents.

A solution of 4:5:7-trihydroxy-2-propylanthraquinone (0·16 g.) in pyridine (6·5 ml.) and acetic anhydride (1·6 ml.) was held at 35° for 3 days. The golden-yellow solution was then poured into water (300 ml.) and the resulting yellow solid (0·18 g.) was crystallized from ethanol. 4:5:7-*Triacetoxy-2-propylanthraquinone* was thus obtained as long, thin, yellow needles, m.p. 182–183°. (Found: C, 64·9; H, 4·8; CH_3CO, 32·7. $C_{23}H_{20}O_8$ requires C, 65·1; H, 4·75; $3CH_3CO$, 30·4%.)

ISOLATION OF NALGIOLAXIN

The dried ground mycelium of *P. nalgiovensis*, after exhaustive extraction with light petroleum (b.p. 40–60°) as described on p. 568, still contains considerable amounts of other substances which can be extracted in part with ether or almost completely with $CHCl_3$. These extractives consist of nalgiolaxin, further amounts of nalgiovensin, other colouring matters, colourless crystalline solids and 'fat'. The fractionation of this complex mixture of extractives presents considerable technical difficulties. The method finally found most suitable, and a typical example of which is given below, was based on the following experimentally established facts. (1) Although pure nalgiolaxin is less soluble in organic solvents than is nalgiovensin, the two colouring matters cannot be satisfactorily separated from each other by fractional crystallization of the crude extractives. (2) Nalgiolaxin and nalgiovensin cannot be separated satisfactorily by chromatography on $MgCO_3$, although by such chromatography they can be separated from the other colouring matters. (3) Nalgiolaxin may be partially separated from nalgiovensin by extraction of an ether or $CHCl_3$ solution with N-Na_2CO_3. (4) Fractional sublimation in a high vacuum can be successfully employed to separate nalgiolaxin from the colourless crystalline solids and traces of nalgiovensin.

The ground mycelium (261 g.), previously extracted exhaustively with light petroleum (b.p. 40–60°), was dried and re-extracted with $CHCl_3$ in five Soxhlets for about 60 hr. During the extraction period a colourless solid (substance *A*) separated in the boiling $CHCl_3$ solution. At the end of the extraction the dark extract was held overnight and substance *A* was then separated by filtration (4·16 g.). The $CHCl_3$ solution was evaporated yielding a brown fatty solid (11·8 g.) from which the fat present (8·0 g.) was removed by extraction with boiling light petroleum (b.p. 40–60°) (0·5 hr., 3 × 500 ml.) and then by macerating with cold ether (100 ml.). There is a slight loss of pigment in the latter step, but it is essential, since otherwise considerable difficulties are experienced with emulsification in subsequent $CHCl_3$ extractions. The brown friable residual solid thus obtained (3·78 g.) was extracted five times with ether (800 ml. each time) by refluxing for 0·5 hr. There was thus obtained an orange-yellow ether solution (*a*) (4 l.), and a brown residue (*b*) (1·33 g.).

The orange-yellow ether solution (*a*) (4 l.) was extracted with four successive portions of aqueous N-Na_2CO_3 (400, 400, 300, 200 ml.). The first extract was an intense wine-red colour. The other extracts were progressively lighter in colour. The Na_2CO_3 solutions were acidified with dil. HCl and the amorphous solid which separated was extracted with $CHCl_3$ (3 × 300 ml.). The $CHCl_3$ solution, on evaporation, yielded a brown solid (1·18 g.) which was crystallized from ethanol (300 ml.) giving crude nalgiolaxin (0·42 g.), m.p. 240–245°, contaminated with traces of nalgiovensin and other substances. It was fractionally sublimed, as described below, to give almost pure nalgiolaxin (0·33 g.), m.p. 245·5–246·5°. Further crops of crystals from the ethanol mother liquor consisted mainly of crude nalgiovensin. The carbonate extracted ether solution, on evaporation to dryness, gave a crystalline orange solid (0·62 g.), m.p. 195–197°, consisting of almost pure nalgiovensin.

The brown residue (*b*) (1·33 g.) was dissolved in $CHCl_3$ (1200 ml.). A small amount of colourless undissolved material was separated by filtration and discarded, and the clear filtrate was passed through a column (3·5 cm. diam.) of $MgCO_3$ (British Drug Houses Ltd., 'heavy'). A total band, 10 cm. in length, was thus formed consisting of a top purple-blue band (3·5 cm.) of an at present uncharacterized colouring matter (substance *B*), and a red band (6·5 cm.). These bands did not spread any further when the column was washed with $CHCl_3$ (400 ml.). The red band was mechanically separated, acidified with 2N-HCl in ice, and the precipitated colouring matter was extracted with $CHCl_3$. On removal of the $CHCl_3$ there remained a brown solid (0·60 g.) which contained considerable amounts of colourless solid. It was distributed equally in five sublimation tubes and was then purified by fractional sublimation in a high vacuum as follows. The oil-bath temperature was raised slowly to 160–170° and was held there for 15 min. A small sublimate of nalgiovensin was thus obtained. The oil bath was lowered and the temperature was raised to 190–200° and held there for 45 min. The resulting orange-red sublimate (0·37 g.), m.p. 241–244°, consisted of crude nalgiolaxin. The unsublimed residue consisted of colourless solid.

Proof that nalgiolaxin is not an artifact

When it became known that nalgiolaxin contains chlorine, but is otherwise closely related to nalgiovensin, it became necessary to prove that it is not formed as an artifact during the process of isolation. This was accomplished by the following method in which no reagents containing chlorine are used. The ground mycelium of *P. nalgiovensis* (160 g.), previously extracted with light petroleum, was extracted in Soxhlets with ether for 39 hr. The orange-yellow solid which separated (0·49 g.) was successively refluxed with small portions of fresh ether (15 × 50 ml.) leaving an undissolved residue (0·41 g.). A portion of this residue (0·10 g.) was dissolved in acetone (350 ml.) at room temperature and the solution was chromatographed on a $MgCO_3$ column (2·5 cm. diam.). A total band of 8·5 cm. was formed consisting of the usual top purple-blue band (0·4 cm.), followed by a red band (6·8 cm.) and then by a yellow band (1·3 cm.) (substance *C*). The column was washed with acetone (100 ml.), and was drained free from acetone. The red band was carefully separated mechanically and was acidified with 2N-H_2SO_4. The mixture was heated on the water bath to coagulate the precipitated solid which was separated by filtration, washed and dried. It was then fractionally sublimed in a high vacuum as described above. The orange sublimate (0·04 g.) obtained at 190–200° melted at 243–244°, raised to 248–248·5° (0·03 g.) by crystallization from ethanol (40 ml.). It did not depress the melting point of pure nalgiolaxin (m.p. 248–248·5°) obtained as described above, where $CHCl_3$ was used as solvent and HCl was used for acidification of the $MgCO_3$ red bands.

Purification of nalgiolaxin

Crude nalgiolaxin, as obtained by the above isolation procedures, was easily purified by crystallization from ethanol. It was usually only necessary to crystallize twice in order to obtain material of constant melting point. In a typical example crude nalgiolaxin (0·37 g.), m.p. 241–244°, was crystallized from ethanol (500 ml.) and gave almost pure material (0·30 g.), m.p. 247–247·5°, raised to a constant m.p. 248–248·5° (0·25 g.) on recrystallization from ethanol.

From 572 flasks which gave 1645 g. of dried mycelium 2·91 g. of nalgiolaxin were obtained. This corresponds to 0·51 g. of nalgiolaxin/100 flasks or 0·18% of the weight of the mycelium. The average yield of nalgiovensin obtained during the isolation of nalgiolaxin was 1·61 g./100 flasks or about 55% of the total yield of nalgiovensin. The total yield of nalgiovensin obtained corresponds to about 1·0% of the weight of the mycelium.

General properties of nalgiolaxin. Derivatives

Nalgiolaxin is pleomorphic in nature and crystallizes from ethanol in small yellow plates and thin needles, m.p. 248–248·5°, without decomposition. (Found, on sample crystallized from ethanol and dried at 100° without loss in wt.: C, 59·8, 59·8; H, 4·2, 4·3; N, S, nil; on sample sublimed in a high vacuum at 190–200°, CH_3O, 8·4; *C*-CH_3, 4·0; Cl, 10·0. $C_{18}H_{15}O_6Cl$ requires C, 59·6; H, 4·2; 1CH_3O, 8·55; 1*C*-CH_3, 4·1; Cl, 9·8%.) It is dextrorotatory in $CHCl_3$ solution: $[\alpha]_{5790}^{22°}$ +40·3° (*c*, 0·1072); the mercury-green line 5461 A. is completely filtered out by the orange-yellow solution. It readily sublimes in a high vacuum at 190–200° without decomposition. It is less soluble in all organic solvents than is nalgiovensin (q.v.). It does not dissolve in 0·5N-$NaHCO_3$ or in N-Na_2CO_3 except on standing, when a pale-red solution with a slight bluish cast is obtained. It dissolves readily in N-NaOH, giving a red solution with a slight bluish cast from which a purplish-red precipitate slowly separates. Its solution in cold conc. H_2SO_4 is red with a much bluer cast than that given by nalgiovensin. Its ethanolic solution gives an orange-brown colour with ethanolic $FeCl_3$ indistinguishable from that given by nalgiovensin and physcion. Its solution in glacial acetic acid is yellow in colour and does not fluoresce in daylight. Its $CHCl_3$ solution gives a red band when chromatographed on columns of $MgCO_3$, the colour being a shade bluer than that given by nalgiovensin and physcion.

Although nalgiolaxin does not dissolve in cold aqueous N-Na_2CO_3 except on standing, it shows a characteristic reaction under the following conditions. Nalgiolaxin (about 2 mg.) was dissolved in $CHCl_3$ (5 ml.) and the yellow solution was vigorously shaken with aqueous N-Na_2CO_3 (5 ml.). On settling, the aqueous phase was light bluish-crimson in colour with a red precipitate at the interface. The $CHCl_3$ solution, which still remained yellow, was re-extracted a further four times with N-Na_2CO_3, each time giving the same light bluish-crimson colour in the aqueous phase with little diminution of the yellow colour in the $CHCl_3$ solution. Under the same conditions nalgiovensin and physcion, in both of which the OH group in position 7 is methylated, gave only an extremely faint crimson colour in the aqueous phase with a yellow colour in the $CHCl_3$ lower layer, whereas emodin, ω-hydroxyemodin and 4:5:7-trihydroxy-2-propylanthraquinone, in all of which the OH group in position 7 is free, gave a deep-red aqueous extract with a slight bluish cast and a colourless $CHCl_3$ lower layer.

Nalgiolaxin (0·05 g.) was dissolved in cold conc. H_2SO_4 (1·8 ml.) containing boric acid (0·13 g.). The intensely red solution was then slowly heated in an oil bath. The colour of the solution took on a bluer shade at 75°, was red-violet at 130° and permanganate-purple at 150–160°. It was held at this temperature for 0·5 hr. and was then cooled, poured on ice, diluted with water (100 ml.) and boiled for 10 min. After cooling, the mixture was extracted with $CHCl_3$ (100 ml.) yielding an orange-pink solution with a fine green fluorescence. On removal of the solvent, the resulting red solid gave the following colour reactions: with cold conc. H_2SO_4, a red-violet solution with a slight red fluorescence; with cold aqueous N-NaOH or N-Na_2CO_3, a blue-violet solution bleaching to colourless overnight; in glacial acetic acid, an orange-yellow solution with a fine green fluorescence. Catenarin (1:4:5:7-tetrahydroxy-2-methylanthraquinone), tested at the same time, gave colours and fluorescent solutions which were very similar to those just described, except that its solution in cold conc. H_2SO_4 was much bluer in tone.

Triacetylnalgiolaxin. Nalgiolaxin (0·27 g.) was acetylated with anhydrous sodium acetate (0·54 g.) and acetic anhydride (6 ml.) in the usual manner. The crude acetate (0·36 g.) was crystallized from ethanol. *Triacetylnalgiolaxin* forms thin lemon-yellow plates (0·29 g.), m.p. 186·5–187°. The same acetate, m.p. and mixed m.p. 186·5–187°, was obtained by acetylating nalgiolaxin in pyridine and acetic anhydride. (Found: C, 58·8; H, 4·3; CH_3O, 6·4; CH_3CO, 25·7; Cl, 6·9. $C_{24}H_{21}O_9Cl$ requires C, 58·95; H, 4·3; 1CH_3O, 6·35; 3CH_3CO, 26·4; Cl, 7·25%.) Triacetylnalgiolaxin is laevorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{20°}$ −7·7°; $[\alpha]_{5790}^{20°}$ −6·1° (*c*, 0·9454).

Pure triacetylnalgiolaxin (0·155 g.) was hydrolysed with aqueous 0·1 N-NaOH (100 ml.) in N_2 as described for the hydrolysis of triacetylnalgiovensin (see p. 568). The recovered nalgiolaxin, crystallized from ethanol and sublimed, had m.p. and mixed m.p. 248–248·5° with a specimen prepared by crystallization from ethanol (see above).

Nalgiolaxin dimethyl ether. A mixture of nalgiolaxin (0·32 g.), anhydrous K_2CO_3 (12·4 g.), dimethyl sulphate (3·2 ml.) and anhydrous acetone (550 ml.) was refluxed for 17 hr. The crude product (0·31 g.) was worked up as described for nalgiovensin dimethyl ether (see p. 568), and was crystallized from ethanol. *Nalgiolaxin dimethyl ether* (0·22 g.) forms thin yellow needles, appearing yellow-green in bulk, m.p. 206–207°. (Found: C, 61·5, 61·4; H, 5·2, 4·6; CH_3O, 23·8; Cl, 8·7. $C_{20}H_{19}O_6Cl$ requires C, 61·5; H, 4·9; $3CH_3O$, 23·8; Cl, 9·1%.) It is dextrorotatory in $CHCl_3$ solution: $[\alpha]_{5461}^{21°} + 27·7°$; $[\alpha]_{5790}^{21°} + 23·5°$ (*c*, 0·4401). It sublimes in a high vacuum at 185–195°. It is insoluble in aqueous N-NaOH, but dissolves in cold conc. H_2SO_4 to an intense red solution with a slight bluish cast.

CrO_3 *oxidation of triacetylnalgiolaxin*

Triacetylnalgiolaxin (0·32 g.) was dissolved in a mixture of glacial acetic acid (6·4 ml.) and acetic anhydride (6·4 ml.) at 55–60°, and oxidized with CrO_3 (0·64 g.) dissolved in water (0·5 ml.) and glacial acetic acid (6·4 ml.). The oxidation procedure and recovery of the oxidation products were similar to those described for the CrO_3 oxidation of triacetylnalgiovensin (see p. 569). The acid fraction, extracted with aqueous 0·5 N-$NaHCO_3$, was obtained as a yellow solid (0·02 g.), m.p. 240–241°. The neutral fraction (0·28 g.) remaining on removal of the $CHCl_3$ was obtained as a yellow oil, partially solidifying on standing, and was oxidized a second time yielding a second acid fraction (0·02 g.) and a neutral fraction (0·22 g.).

The combined acid fractions (0·04 g.) were crystallized from ethanol (5 ml.). *Diacetylchloroemodic acid 7-methyl ether* was thus obtained as yellow microplates (0·03 g.), m.p. 241–242°. (Found: C, 55·4, 55·6; H, 3·4, 3·3; Cl, 8·6. $C_{20}H_{13}O_8Cl$ requires C, 55·5; H, 3·0; Cl, 8·2%.) It dissolves in 0·5 N-$NaHCO_3$ to a reddish-yellow solution, whereas diacetylemodic acid 7-methyl ether gives a pure yellow solution. On the other hand, their solubilities and colour reactions in N-Na_2CO_3, N-NaOH and cold conc. H_2SO_4 are indistinguishable from each other.

The neutral fraction (0·22 g.) obtained from the second oxidation was crystallized from ethanol (125 ml.). The *product* (0·17 g.) was thus obtained as lemon-yellow plates and needles, m.p. 216–217°. (Found: C, 57·3, 57·3; H, 4·0, 4·1; CH_3O, 6·7. $C_{22}H_{17}O_9Cl$ requires C, 57·3; H, 3·7; $1CH_3O$, 6·7%.) It differs from triacetylnalgiolaxin (m.p. 186·5–187°) in melting point and in the fact that it is optically inactive in $CHCl_3$ solution (*c*, 0·4337). It is insoluble in cold N-NaOH, gives no colour with ethanolic $FeCl_3$ and dissolves in cold conc. H_2SO_4 to a red solution with a very slight bluish cast.

Reduction of nalgiolaxin

A mixture of nalgiolaxin (0·18 g.), glacial acetic acid (5 ml.), red phosphorus (0·25 g.) and HI (1 ml., sp.gr. 1·7) was refluxed for 5 hr. The resulting anthranol was isolated as described in the reduction of nalgiovensin (see p. 571), and was also reduced a second time. The crude yellow anthranol (0·135 g., m.p. 167–168°), dissolved in glacial acetic acid (150 ml.) at 60°, was oxidized to the corresponding anthraquinone with CrO_3 (0·13 g.) dissolved in water (5 ml.) and glacial acetic acid (5 ml.). The crude oxidation product (0·12 g.) was purified by crystallization from $CHCl_3$, followed by sublimation in a high vacuum at 170–175° and crystallization of the sublimate from benzene. It was thus obtained as characteristic long yellow needles (0·04 g.), m.p. 216·5–217°, not depressed on admixture with 4:5:7-trihydroxy-2-propylanthraquinone (m.p. 216·5–217°) obtained by reduction of nalgiovensin. The two products also gave identical colour reactions. On acetylation in pryidine and acetic anhydride, 4:5:7-triacetoxy-2-propylanthraquinone was obtained. It melted at 182–183°, not depressed on admixture with 4:5:7-triacetoxy-2-propylanthraquinone (m.p. 182–183°) obtained from nalgiovensin.

Other products extracted from the mycelium of Penicillium nalgiovensis

As mentioned in previous sections the mycelium of *P. nalgiovensis* contains three other substances, *A*, *B* and *C*, which can be extracted with solvents. None of these substances has been characterized, but the following is a summary of what is known of them at present.

Substance A. This substance (see p. 571) separated from the boiling $CHCl_3$ solution and is present in considerable amounts, averaging about 5 g./100 flasks. It crystallizes from glacial acetic acid as fine colourless microneedles which sublime readily in a high vacuum at 250–260°, and on heating in a capillary tube sublime at about 300° without melting. It is only slightly soluble in glacial acetic acid, $CHCl_3$, acetone, benzene, ethanol and ethyl acetate. It is insoluble in water, 2 N-HCl and 2 N-NaOH. It gives no colour with ethanolic $FeCl_3$.

Substance B. This substance is a colouring matter which is present in the top purple-blue band during chromatography on $MgCO_3$ of the $CHCl_3$ extract (see p. 571). The crude colouring matter recovered by acidification of the separated purple-blue band gives the following colour reactions. It dissolves in cold conc. H_2SO_4 giving an intensely coloured solution which appears red-violet by transmitted light and blue-violet by reflected light. It readily dissolves in N-Na_2CO_3 and N-NaOH, giving in each case a red-violet colour. Its solution in glacial acetic acid is orange-yellow in colour and does not fluoresce in daylight. It appears to be quinonoid in nature and may be a polyhydroxyanthraquinone.

Substance C. This substance is present in the bottom yellow band during chromatography on $MgCO_3$ of the ether extract (see p. 572), and is present in only very small amounts. The yellow band begins to change colour quickly on exposure to air passing through brown to purple. The crude material recovered by immediate acidification of the separated yellow band gives the following colour reactions. It dissolves in cold conc. H_2SO_4 giving a greenish solution which becomes an intense dark green on standing. It is insoluble in N-Na_2CO_3 but readily dissolves in N-NaOH to a yellowish solution which, on shaking, quickly changes colour giving finally a red solution with a slight bluish cast. Substance *C* appears to be an anthranol, probably of the emodin-anthranol type, since a specimen of 4:5:7-trihydroxy-2-propylanthranol behaves very similarly on chromatography on $MgCO_3$ and gives almost identical colour reactions (see p. 571).

SUMMARY

1. Two hitherto undescribed colouring matters, *nalgiovensin* and *nalgiolaxin*, have been isolated from the mycelium of laboratory cultures of *Penicillium nalgiovensis* Laxa.

2. Nalgiovensin, $C_{18}H_{16}O_6$, crystallizes in orange needles or plates, m.p. 199–200°. It has been shown to be *dextro*-4:5-dihydroxy-7-methoxy-2-hydroxypropylanthraquinone in which the nature of the hydroxypropyl group, whether —CHOH.CH_2.CH_3, —CH_2.CHOH.CH_3 or —CH(CH_3).CH_2OH, is at present undetermined.

3. Nalgiolaxin, $C_{18}H_{15}O_6Cl$, crystallizes in yellow needles or plates, m.p. 248–248·5°. It has been shown to be a monochloroderivative of nalgiovensin and is believed to be *dextro*-1(or 8)-chloro-4:5-dihydroxy-7-methoxy-2-hydroxypropylanthraquinone.

4. Nalgiolaxin is believed to be the first example of a derivative of anthraquinone containing chlorine which has been recorded from any natural source.

5. Nalgiovensin and nalgiolaxin are both dextrorotatory in chloroform solution and are of unusual interest since optically active, naturally occurring, derivatives of anthraquinone are rare except those which occur as glycosides.

REFERENCES

Anslow, W. K., Breen, J. & Raistrick, H. (1940). *Biochem. J.* **34**, 159.

Anslow, W. K. & Raistrick, H. (1941). *Biochem. J.* **35**, 1006.

Charles, J. H. V., Raistrick, H., Robinson, R. & Todd, A. R. (1933). *Biochem. J.* **27**, 499.

Eder, R. & Hauser, F. (1925). *Helv. chim. Acta*, **8**, 126, 140.

Fischer, O. & Gross, H. (1911). *J. prakt. Chem.* **84**, 369.

Fuson, R. C. & Bull, B. A. (1934). *Chem. Rev.* **15**, 275.

Hayashi, M. (1927). *J. chem. Soc.* p. 2516.

Jacobson, R. A. & Adams, R. (1924). *J. Amer. chem. Soc.* **46**, 1312.

Laxa, O. (1932). *Zbl. Bakt.* (2 Abt.), **86**, 160.

Oesterle, O. A. (1911). *Arch. Pharm., Berl.*, **249**, 445.

Purdie, T. & Irvine, J. C. (1901). *J. chem. Soc.* **79**, 960.

Purdie, T. & Irvine, J. C. (1903). *J. chem. Soc.* **83**, 1021.

Raistrick, H., Robinson, R. & Todd, A. R. (1934). *Biochem. J.* **28**, 559.

Raper, K. B. & Thom, C. (1949). *A Manual of the Penicillia*, p. 319. Baltimore: Williams and Wilkins.

Ullmann, F. & Schmidt, W. (1919). *Ber. dtsch. chem. Ges.* **52**, 2098.

The Influence of a High-Sucrose Diet on the Calcium and Phosphorus Percentage of the Rat Femur, and a Comparison with its Effect on the Enamel and Dentine of the Rat Incisor Teeth

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The enamel and dentine of the incisors of rats maintained on a diet containing 67% sucrose contained significantly higher percentages of calcium and phosphorus than the same tissues of animals maintained on a stock diet of Lever cubes (Hartles, 1951). The calcium/phosphorus ratios on the high-sugar diet were, however, significantly lower than those on the stock diet showing that there was a relatively greater increase in phosphorus than in calcium.

The left femur of each of these animals was removed at the same time as the teeth and kept for subsequent analysis. The work reported here is concerned with the effect of the high-sugar diet on the percentage of calcium and phosphorus in these bones, and includes a comparison with the effect on the incisor teeth. The results show that in the femur there is no significant difference in the calcium and phosphorus percentage or the calcium/phosphorus ratios on the two diets. The high-sugar diet, therefore, affected the incisor teeth but not the bone.

EXPERIMENTAL

Animals and diet. The animals and diets were those used in the tooth study (Hartles, 1951): group I, twenty-two animals maintained on a diet of Lever rat cubes; group II, twenty-seven animals weaned at 21 days on to the diet containing 67% sucrose.

The animals were killed with ether, the left femora removed at the same time as the teeth and placed in absolute ethanol for 48 hr. The atomic Ca/P ratio of the stock diet was 1·36 and of the high-sugar diet 1·35.

Treatment of bones. Each femur was stripped of adhering tissue, broken into small fragments, wrapped in a small filter-paper parcel and placed in absolute ethanol overnight.

The fragmented femora were then extracted with ether for 6 hr. and dried at 110° for 3 hr. The dry, fat-free bones were pulverized in a percussion mortar so as to pass through a 60-mesh wire sieve. Tissue debris and non-mineralized tissue were removed from the powdered bone by flotation in a bromoform-acetone mixture of sp.gr. 1·5. This is admittedly a somewhat arbitrary treatment, but it was found that material separated up to sp.gr. 1·5 and not in any quantity above that value. The procedure has the merit of producing a standard material for analysis. In this report the analytical figures for bone refer to that portion of the dry, fat-free femur of density greater than 1·5.

Analytical procedure. Samples of bone (50–100 mg.) were added to 10 ml. of 5 N-HCl and allowed to stand for 1 hr. The mixture was made alkaline with ammonia, re-acidified to pH 5·2 with acetic acid and made up to 50 ml. The acid-insoluble residue was removed by filtration, and calcium and phosphorus determinations were carried out on the filtrates as previously described (Hartles, 1951).

RESULTS

The results for the calcium and phosphorus determinations and the calcium/phosphorus ratios are given in Table 1, together with those for dentine and enamel for comparison. It is interesting to note that the calcium/phosphorus ratio for the rat femur approximates to that of the incisor enamel rather than the dentine.

Table 1. *Percentages of calcium and phosphorus in the dry fat-free femur and incisor teeth of rats on a stock diet and a high sucrose* (67 %) *diet*

(Tooth results reproduced from Hartles, 1951.)

	Ca (%) Mean ± S.E.M.	P (%) Mean ± S.E.M.	Ca/P atomic ratio Mean ± S.E.M.
Stock diet:			
Femur	25·41 ± 0·18	12·25 ± 0·13	1·608 ± 0·016
Dentine	26·00 ± 0·29	13·54 ± 0·16	1·486 ± 0·012
Enamel	33·17 ± 0·37	15·99 ± 0·15	1·607 ± 0·014
High-sugar diet:			
Femur	25·57 ± 0·22	12·21 ± 0·07	1·623 ± 0·014
Dentine	26·75 ± 0·23	14·57 ± 0·12	1·425 ± 0·014
Enamel	35·06 ± 0·31	17·27 ± 0·11	1·569 ± 0·012

There was no significant difference between the percentages of calcium and phosphorus in the dry, fat-free femora of rats maintained on the stock diet and of those maintained on the high-sucrose diet. Neither was there any significant difference in their calcium/phosphorus ratios.

This finding is in contrast to the results for the enamel and dentine of the incisor teeth of the same animals (Hartles, 1951).

DISCUSSION

In most experiments conducted on bone and teeth it has been found relatively easier to influence the composition of bone than that of the developing tooth. For example, rachitogenic diets produce only slight changes in the teeth of dogs (Smith & Light, 1945). Clarke & Smith (1935) showed that rats maintained on diets almost completely lacking in minerals still continued to deposit small amounts of minerals in the incisor teeth, although the bones were greatly altered. Any conditions, therefore, which appear to affect the developing tooth rather than the bone are of interest. Gaunt & Irving (1940) showed that large variations in the calcium/phosphorus ratio of the diet affected the incisor teeth and bone of rats in a different way. When the calcium/phosphorus ratio was 4·0 much better teeth were formed than at a ratio of 0·5. The effect on the bone was the exact converse; at a calcium/phosphorus ratio of 4·0 the bones were severely rachitic, whilst at the lower ratio of 0·5 bone of more normal structure was formed. The present work shows that the high-sugar diet which affects the composition of the enamel and dentine does not change that of the bone.

In view of this finding it is of interest to study the degree of correlation between the amounts of calcium and phosphorus in the three tissues.

Teeth. On the stock diet there is a high degree of correlation between the calcium in enamel and dentine and the phosphorus in enamel and dentine (the probability that the correlation is due to chance being 1 in 50 and < 1 in 100, respectively), i.e. if the calcium and phosphorus contents are high in the enamel there is a strong probability that they will be high in the dentine and vice versa.

On the sugar diet, however, the probability of high dentine calcium being associated with high enamel calcium is very small, and there is no correlation between dentine and enamel phosphorus. Values for the partial correlation coefficients are given in Table 2.

Teeth and bone. On the stock diet values calculated for the correlation coefficient show no correlation between calcium or phosphorus in enamel and bone, and for dentine and bone there is correlation only for phosphorus (the probability of its being due to chance is < 1 in 100).

Table 2. *Values of partial correlation coefficients for calcium in enamel, dentine and bone, and phosphorus in enamel, dentine and bone*

	Calcium		Phosphorus	
	Dentine	Enamel	Dentine	Enamel
		Stock diet		
Bone	0·176	−0·072	0·458*	−0·198
Dentine	—	0·488*	—	0·650*
		$n-3=19$		
		Sugar diet		
Bone	−0·269	−0·278	−0·297	0·388*
Dentine	—	0·269	—	0·116
		$n-3=24$		

* Significant correlation.

Table 3. *Values of correlation coefficients between calcium and phosphorus in enamel, dentine and bone*

	Stock diet Calcium			Sugar diet Calcium		
	Enamel	Dentine	Bone	Enamel	Dentine	Bone
Phosphorus:						
Enamel	0·628	—	—	0·482	—	—
Dentine	—	0·629	—	—	0·276	—
Bone	—	—	0·435	—	—	0·282
		$n-2=20$			$n-2=25$	

On the sugar diet for enamel and bone there is a doubtfully significant negative correlation for calcium and a significant positive correlation for phosphorus (the probability of its being due to chance is <1 in 20). For dentine and bone there are doubtfully significant negative correlations for both calcium and phosphorus. Values for the partial correlation coefficients are given in Table 2.

The calcium/phosphorus ratio. This ratio is widely used in investigations on teeth and bone and it is important to know, therefore, the degree of correlation between calcium and phosphorus in any of the tissues.

On the stock diet there are high degrees of correlation between calcium and phosphorus content in enamel, dentine and bone, especially in the two former tissues (the probability of their being due to chance are respectively <1 in 100, <1 in 100 and <1 in 20). The effect of the high-sugar diet is to reduce the significance of the correlation in enamel and to render the figures for dentine and bone doubtfully significant. The values for the correlation coefficients are given in Table 3.

It can be concluded from the above data that on a nutritionally sound laboratory stock diet there is a high degree of correlation between enamel and dentine calcium and phosphorus content in the rat incisor, even though the calcium/phosphorus ratios of the two tissues may differ (Sobel & Hanok, 1949; Hartles, 1951). The effect of the high-sugar diet is to lessen markedly this correlation, particularly in the case of phosphorus.

Enamel is ectodermal and dentine mesodermal in origin; even so, if the processes leading to the deposition of minerals were identical in enamel and dentine it would be expected that alteration of the dietary regimen should affect both tissues equally.

Murray (1936) has pointed out that magnesium can replace calcium in a mineralized tissue to a certain extent. This may be a reason why in the cases of enamel and bone, and dentine and bone, on the stock diet the only significant correlation is between dentine phosphorus and bone phosphorus. In the bone, itself, the sucrose diet has not significantly altered the mean values for calcium, phosphorus or the calcium/phosphorus ratio, but the correlation between calcium and phosphorus is reduced to doubtful significance.

Murray (1936) found wide differences in the composition of the mineral constituents of whole teeth in different animals, in different types of teeth in the same animal, and in bones and teeth of the same animal and in different tissues of the same tooth. She suggests that mineralization is an active and specific cell process and not merely a precipitation reaction. Experiments with radioactive isotopes (^{32}P) have confirmed the dynamic nature of bone (Chievitz & Hevesy, 1935, 1937); similar experiments with teeth have shown that ^{32}P is taken up but to a lesser degree (Manley & Bale, 1939). The continually growing rat incisor shows greatest uptake of the isotope at the root, and as the tooth grows the amount in the outer portions increases. The isotope also enters the molar teeth, but the uptake is only one-third of that in the incisor.

There is therefore enough evidence to show that the minerals of the teeth are not static. The apparent stability of the minerals of the teeth may be due to a dynamic state in which the equilibrium is much more resistant to change than is the case in bone, although it must be remembered that bone has a much greater blood supply than teeth.

The enzyme now known as alkaline bone phosphatase was first observed by Robison & Soames (1924); it is present in both teeth and bone. In enamel, the enzyme does not actually occur within the ameloblast but outside in the stratum intermedium (Bevelander & Johnson, 1945; Morse & Greep, 1947*a*, *b*). On the other hand, phosphatase occurs actually within the odontoblast and the adjacent pulp during mineralization of the dentine (Bevelander & Johnson, 1946). Histochemical methods have revealed that the ameloblasts contain more phosphorus than do the odontoblasts (Hintzsche & Baumann, 1933). By the use of micro-incineration techniques Hampp (1940) showed that the mineral content of the ameloblasts differed considerably from that of the odontoblast. There is therefore some evidence to suggest that the ameloblasts and odontoblasts differ chemically and biochemically.

The influence of the high-sugar diet on the enamel, dentine and bone of the rat lends support to the view that the processes of mineralization in the three tissues are significantly different.

SUMMARY

1. The calcium and phosphorus content and the calcium/phosphorus ratios in the rat femur were the same whether the animals were raised on a stock diet of Lever cubes or a diet containing 67 % sucrose.

2. This is in contrast to the effect on the enamel and dentine of the incisor teeth of the same rats (Hartles, 1951), and makes it clear that the 'sugar' disturbance is confined to the teeth and does not include bone.

3. The correlation between the amounts of calcium and phosphorus in enamel, dentine and bone has been evaluated.

4. The findings support the view that the processes of mineralization in the three tissues are significantly different.

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REFERENCES

Bevelander, G. & Johnson, P. L. (1945). *J. cell. comp. Physiol.* **26**, 25.
Bevelander, G. & Johnson, P. L. (1946). *J. dent. Res.* **25**, 381.
Chievitz, O. & Hevesy, G. (1935). *Nature, Lond.*, **136**, 754.
Chievitz, O. & Hevesy, G. (1937). *K. danske vidensk. Selsk. Skr.* **13**, no. 9. Cited in *Chem. Abstr.* (1937), **31**, 4711.
Clarke, M. F. & Smith, A. H. (1935). *Amer. J. Physiol.* **112**, 286.
Gaunt, W. E. & Irving, J. T. (1940). *J. Physiol.* **99**, 18.
Hampp, E. G. (1940). *Anat. Rec.* **77**, 273.
Hartles, R. L. (1951). *Biochem. J.* **48**, 245.
Hintzsche, E. & Baumann, M. (1933). *Z. mikr.-anat. Forsch.* **32**, 333.
Manley, M. L. & Bale, W. F. (1939). *J. biol. Chem.* **129**, 125.
Morse, A. & Greep, R. O. (1947*a*). *Anat. Rec.* **97**, 357.
Morse, A. & Greep, R. O. (1947*b*). *Anat. Rec.* **99**, 379.
Murray, M. M. (1936). *Biochem. J.* **30**, 1567.
Robison, R. & Soames, K. M. (1924). *Biochem. J.* **18**, 740.
Smith, C. R. H. & Light, A. H. (1945). *J. dent. Res.* **24**, 53.
Sobel, A. E. & Hanok, A. (1949). *J. biol. Chem.* **176**, 1103.

Studies on the Metabolism of the Protozoa

1. METABOLISM OF THE PARASITIC FLAGELLATE, *TRYPANOSOMA LEWISI*

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Trypanosoma lewisi has its natural environment in the blood stream of the rat. In this medium, oxygen, glucose and carbon dioxide are readily available to meet the energy requirements of the flagellate. Biot, Biot & Richard (1911) first indicated the important part played in the life of *T. lewisi* by glucose, which according to Reiner, Smythe & Pedlow (1936) is metabolized with the production of acid. Searle & Reiner (1941) showed that under aerobic conditions, almost 50 % of this acid was acetic acid, and stated that carbon dioxide had a pronounced stimulatory effect on the respiration. Under anaerobic conditions, they found that very little glucose was used, unless pyruvate or carbon dioxide was also present. In this latter case, they demonstrated the fixation of carbon dioxide, with the production of succinic, lactic, pyruvic and acetic acids. Moulder (1948*a*, *b*) showed that, in the adult phase, *T. lewisi* was able to oxidize glucose more efficiently than in the reproductive phase, and that the trypanosome was also

capable of oxidizing other substrates. In the present study, some of these results are confirmed and extended. New observations are reported concerning the aerobic and anaerobic rates of glucose utilization by *T. lewisi*, and results described of a quantitative study of the various metabolic products. The aerobic metabolism of *T. lewisi* is shown to depend on the cytochrome system. Experiments are described on the action of a variety of inhibitors, including a light-reversible inhibition by carbon monoxide.

MATERIAL AND METHODS

Trypanosome suspensions. The strain of *T. lewisi* used was isolated in 1945 by Dr P. Tate of the Molteno Institute, Cambridge, from a wild stack rat at Grantchester, and has been maintained in hooded or albino laboratory rats ever since, intraperitoneal or subcutaneous inoculations being made at least every 2 weeks. The infestation reaches a maximum of $1–3 \times 10^5$ flagellates/μl. blood on the 6th day of infection, and then slowly declines. The organisms used in this investigation have been harvested at 10–12 days, at an age corresponding to the so-called 'adult' phase of Taliaferro. Infected rats (about 200 g. body weight) were anaesthetized with ether, tied down, and the left interior carotid artery dissected out. A section of this was clipped off, a glass cannula inserted into the artery under a dissecting microscope, and on removing the clip, the blood was collected in 5 ml. of a 0·9% solution of NaCl containing 1% sodium citrate. In this manner about 8·5 ml. blood could be obtained from a 200 g. animal, the process taking about 12 min. Pooled samples of blood were centrifuged for 10 min. at 4000 rev./min. and the supernatant discarded. Most of the trypanosomes separated as a white layer above the red blood cells. They were collected with a Pasteur pipette, along with some red cells. The remaining red cells were twice suspended in a 0·9% solution of NaCl containing 1% glucose (glucose-saline), centrifuged, and each time the upper trypanosome layer was collected. The resulting preparation contained most of the trypanosomes, but only about one-thirtieth of the original red cells. The material containing the trypanosomes was diluted with glucose-saline, and the remaining erythrocytes removed by means of an agglutinating antiserum to rat erythrocytes, obtained from rabbits treated by the method of Moulder (1948*a*). Sufficient of this antiserum was added to the trypanosome suspension to agglutinate all the red cells. The mixture was incubated at 37° for 15 min. and the agglutinated blood corpuscles removed by centrifugation for 1 min. at a low speed. The supernatant containing the trypanosomes was then centrifuged at a higher speed, the trypanosomes washed two or three times, and made up into a suspension with a N content of 0·5–1 mg. N/ml. The suspending medium was either a Ringer-bicarbonate solution containing 0·1 M-NaCl, 0·007 M-KCl, 0·002 M-$MgSO_4$ and 0·016 M-$NaHCO_3$, which in the presence of 5% CO_2 in the gas phase has a pH of 7·3; or a Ringer-phosphate solution containing 0·094 M-NaCl, 0·005 M-KCl, 0·001 M-$MgSO_4$ and 0·025 M-phosphate (added in the form of sodium phosphate buffer, pH 7·3). The preparations of *T. lewisi* obtained by the above method were free from both erythrocytes and leucocytes, but often contained small clusters of blood platelets. 10^9 Protozoal cells are equivalent to about 8·8 mg. material (dry wt.), or 1 mg. N. This quantity of material can usually be obtained from the blood of a single rat. The results of measurements on the metabolic activities of trypanosomes were based on the N content of the suspensions, and expressed as q_{O_2} and $q^{N}_{CO_2}$, i.e. as μl. gas/mg. N/hr.

Spectroscopic observations on cytochrome were made by means of a microscope fitted with a Zeiss microspectroscope ocular. Observations were made both at room temperature, and at low temperatures, as described by Keilin & Hartree (1949, 1950).

Metabolic experiments. These were carried out at 37° in Warburg manometers, in cups (volume 20–25 ml.) fitted with two side arms. Gas phases of 5% CO_2–95% O_2, 5% CO_2–95% N_2, or air were used. The effect of CO on respiration was studied at 20° in Barcroft differential manometers. The source of light used to reverse the inhibition caused by CO was a mercury vapour lamp. The decrease in bicarbonate concentration in the medium during glucose metabolism gave a measure of the total amount of acid produced. Initial and final estimations of bicarbonate in the medium were made by tipping in 0·2 ml. 2 N-H_2SO_4 from a side bulb, and measuring the CO_2 expelled. The difference between the decrease in bicarbonate concentration and the CO_2 actually liberated during the period of glucose metabolism, gives a measure of the CO_2 formed or fixed. The manometer contents were then neutralized with 0·2 ml. 2 N-NaOH, and samples taken for estimation of the various metabolites.

Estimation of metabolites. Glucose was estimated iodometrically by the method of Somogyi (1945) in $Ba(OH)_2$-$ZnSO_4$ filtrates, or when very small samples only were available, in neutralized trichloroacetic acid filtrates by the method of Folin & Malmros, as described by Umbreit, Burris & Stauffer (1945). The method was modified by using 0·5% gelatin as stabilizing colloid in place of 2% gum ghatti, the colour being measured after 30 min. Gelatin has a zeta-potential about one-twentieth that of the various gums, and was found to be much more efficient in preventing the precipitation of the Prussian blue. Using trichloroacetic acid filtrates, pyruvic acid was estimated colorimetrically by the method of Friedemann & Haugen (1943), lactic acid by the colorimetric method of Barker & Summerson, as described by Umbreit *et al.* (1945), and citric acid by the method of Speck, Moulder & Evans (1946). Formic acid was estimated colorimetrically by the method of Grant (1947) on a sample of a distillate from a tungstic acid filtrate. Acetic acid was estimated in another sample of this distillate, by redistilling it from HgO, and titrating the distillate as described by Buchanan, Sakami, Gurin & Wilson (1947). Ethanol was estimated by the bichromate method of Nicloux, le Breton & Doutcheff (1934). The aniline blue spot test of Feigl (1937) was used to test for oxalic acid. Succinic acid was estimated manometrically by means of a washed horse-heart preparation of succinic oxidase. A preliminary ether extraction was not used, because it was found that none of the other metabolites present gave an O_2 uptake with the enzyme preparation, and succinic acid recoveries were the same as after prolonged ether extraction.

RESULTS

Aerobic metabolism

Under aerobic conditions in the presence of utilizable substrate, the trypanosomes are highly motile. In the absence of substrate, the organisms quickly

become immotile, but the motility can be restored by the addition of glucose, provided that the period without substrate has not been too long. Anaerobically, in the presence of glucose, the trypanosomes show a lower motility than under aerobic conditions, but this motility can be restored to normal by the admission of air.

Respiratory activity. A suspension of washed trypanosomes, in Ringer-phosphate solution in air, respires at a low, but steady rate over a period of several hours, with a q_{O_2} of about 40. However, several substances, when added to a washed suspension of trypanosomes, are able to stimulate this respiration over tenfold, and to restore the motility of the flagellates. In the presence of glucose, which is the oxidizable substrate the trypanosomes find *in vivo*, they respire with a q_{O_2} of about 600. On this level, the respiration continues at a constant rate over a period of an hour or two (Fig. 1). Using air as

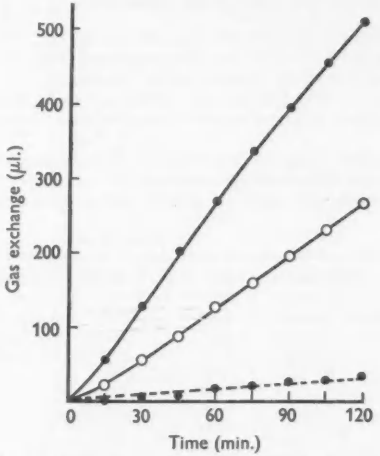

Fig. 1. Aerobic and anaerobic metabolism of glucose by *T. lewisi*. Each manometer cup contained 0·02 M-glucose and 0·48 mg. trypanosome N (approx. 5×10^8 cells) in a volume of 1·5 ml.; temp. 37°; ●——●, O_2 uptake (μl.) in presence of glucose; ● - - - - - - ●, O_2 uptake (μl.) in absence of glucose; ○——○, anaerobic CO_2 production (μl.) in bicarbonate buffer.

the gas phase, the ability of thirty-three different substances to stimulate respiration was tested. The substrate was added from a side bulb after the period of manometric equilibration to give a final concentration of 0·02 M, and respiration was followed over a period of 1 hr. or more. The substances examined fall into three groups:

(*a*) Glucose, mannose and fructose are readily oxidized with a q_{O_2} of about 600. Glutamine is oxidized with the liberation of free ammonia, with a q_{O_2} of 730.

(*b*) Glycerol is oxidized with a q_{O_2} of about 250, and asparagine and glutamic acid with a q_{O_2} of about 100.

(*c*) The following substances failed to increase respiration above the endogenous level, or support motility: galactose, sorbose, xylose; maltose, sucrose, lactose; mannitol, sorbitol, dulcitol, inositol; gluconic acid; DL-glyceraldehyde, dihydroxyacetone, pyruvate, lactate, ethylene glycol, ethanol; citrate, succinate, α-keto glutarate; formate, acetate, proprionate; glycine, alanine and aspartic acid. This list contains, among other substances, lactate and succinate, which are both known to be formed normally by *T. lewisi* during its anaerobic glucose metabolism. Additional evidence that these substances are not utilized aerobically by the protozoa, is shown by the following experiment. A suspension of washed trypanosomes was incubated anaerobically with glucose until all the sugar had been fermented. At this point, air was admitted to the incubated mixture. There was no significant oxygen uptake, nor was there any aerobic disappearance of lactic acid, which had accumulated during the anaerobic phase. In spite of the inability of lactate and succinate to increase the respiration, the trypanosomes are not devoid of the lactic and succinic dehydrogenases, as shown by experiments on homogenized cells (Table 1 and Fig. 4).

Table 1. *Succinic and lactic dehydrogenase activity in* Trypanosoma lewisi *homogenate*

(Homogenate prepared by shaking an aqueous suspension of trypanosomes with glass beads in Mickle disintegrator for 5 min. Phosphate buffer (pH 7·3) added to final concn. of 0·05 M. Thunberg tubes used, each containing 1·16 mg. trypanosome N, 0·03 M-substrate, and 0·134 μmol. methylene blue, added from a hollow stopper; total vol. 2·5 ml. Tubes evacuated and washed with pure N_2 six times. Temp. 37°.)

	Time for decolorization (min.)	
	90%	100%
Homogenate	>180	>180
Homogenate + succinate	37	51
Homogenate + lactate	42	62

Products of glucose oxidation. Using the indirect method of Warburg, determinations were made of the respiratory quotient and acid production during glucose oxidation. A manometer vessel of 13 ml. capacity, containing 5 ml. fluid, was used in conjunction with another of 23 ml. capacity, containing 1·2 ml. fluid. In each flask, the tissue/fluid and glucose/fluid ratios were the same. The organisms were suspended in a bicarbonate medium, and the carbon dioxide-oxygen gas phase was used. Initial and final acid tips were used to determine acid production, and initial and final glucose determinations were made by the iodometric method. Three

experiments gave R.Q. values of 0·94, 1·00 and 0·96 (mean 0·97), and showed that 1 mol. of glucose is oxidized by 3·17 mol. oxygen, to liberate 1·19 equiv. of acid and 3·06 mol. carbon dioxide. Using the direct method of Warburg with a phosphate buffer, the oxygen/glucose ratio (not corrected for the blank oxygen uptake) was 3·86 (an average of nine values, ranging from 3·51 to 4·18). An attempt was made to analyse completely the overall reaction occurring during glucose oxidation. The experiment was carried out in five manometers, the combined contents of which were analysed as indicated in Table 2. In the same table, a carbon and acid balance sheet for the oxidation is given.

Effect of inhibitors on respiration and motility. The sensitivity of respiration to a number of inhibitors was studied; the results are given in Table 3. In most cases the trypanosomes were allowed to respire in the main vessel of the manometer in the presence of 0·01 M-glucose for about 20 min. and then the inhibitor was tipped in from the side arm. In the case of cyanide, balanced potassium hydroxide-potassium cyanide mixtures were used in the centre well as recommended by Robbie (1946). 0·02 M-Fluoride had no observable effect on the motility of the trypanosomes, and only a small effect on respiration. 0·001 M-2:4-Dinitrophenol, which had approximately the same effect on respiration as 0·02 M-fluoride, reduced the motility practically to zero; motility was restored to normal on washing. 0·001 M-Cyanide rendered the trypanosomes almost immotile, but activity was restored if the hydrogen cyanide was removed. 0·0001 M-Iodoacetate, over a period of 45 min., gradually reduced the motility and respiration of the flagellates to zero; this effect was found to be irreversible. Carbon monoxide

Table 2. *Metabolic products formed during aerobic and anaerobic glucose breakdown*

(Each manometer flask contained 0·68 mg. trypanosome N, 4 mg. glucose added from side arm after equilibration, 0·016 M-bicarbonate; total vol. 3·0 ml.; temp. 37°; gas phase 5% CO_2–95% O_2 or 5% CO_2–95% N_2 (five flasks each). After 180 min. incubation, 0·4 ml. 2 N-H_2SO_4 added from second side bulb to stop reaction and to estimate residual bicarbonate. Contents of five flasks neutralized, pooled, and samples taken for estimation of the various metabolites. Yields expressed in terms of mol. metabolite found per mol. glucose disappearing, from which values for carbon and acid recoveries have been calculated.

During the aerobic reaction, a total of 81·7 μmol. glucose was used (by 3·4 mg. trypanosome N). Previous experiments had shown that for 1 mol. glucose oxidized, 3·17 mol. O_2 were used, and 3·06 mol. CO_2 were produced.[1]

During the anaerobic reaction, a total of 72·2 μmol. glucose was used. For each mol. glucose used, a net fixation of 0·74 mol. CO_2 was observed.)

	Aerobically			Anaerobically		
Metabolite	Mol.	C atoms	Acid equiv.	Mol.	C atoms	Acid equiv.
Carbon dioxide	3·06	3·06	—	—	—	—
Succinic acid	0·18	0·72	0·36	0·89	3·56	1·78
Lactic acid	0·12	0·36	0·12	0·39	1·17	0·39
Pyruvic acid	0·03	0·09	0·03	0·19	0·57	0·19
Acetic acid	0·47	0·94	0·47	0·18	0·36	0·18
Ethanol	0·05	0·10	—	0·02	0·04	—
Formic acid	0·00	—	—	0·00	—	—
Oxalic acid	0·00	—	—	0·00	—	—
Citric acid	0·00	—	—	0·00	—	—
Sum	—	**5·27**	**0·98**	—	**5·70**	**2·54**
C atoms supplied (as glucose and CO_2)	—	6·00	—	—	6·74	—
Acid yield (from bicarbonate used)	—	—	1·19	—	—	2·50

Table 3. *The effect of various inhibitors on respiration*

(Each manometer vessel contained about 0·4 mg. trypanosome N in a Ringer-phosphate medium, pH 7·3. Glucose concentration 0·01 M. Total vol. 2·0 ml.; temp. 37°; gas phase air, with 0·2 ml. 20% KOH in centre well. O_2/glucose ratio represents the number of mol. O_2 utilized/mol. glucose disappearing. Figures in brackets represent the ratio in absence of inhibitor.)

Inhibitor	Concentration (M)	Inhibition (%)	O_2/glucose ratio
2:4-Dinitrophenol	0·001	24	—
Sodium fluoride	0·02	18	—
Sodium arsenite	0·0001	59	2·58 (3·54)
Potassium cyanide	0·00001	52	2·26 (4·05)
Sodium azide	0·001	27	3·13
Sodium azide	0·005	58	2·04
Hydroxylamine	0·0004	18	4·00 (4·13)
Hydroxylamine	0·002	56	2·48
Carbon monoxide	95% in gas phase	38	—

(95% in the gas phase) inhibited the respiration by 38%, and the effect was found to be reversible by light (Fig. 2). Disappearance of glucose in the presence of cyanide, azide, hydroxylamine and arsenite, was not inhibited to the same extent as oxygen uptake. A comparison of data in Tables 3 and 5, shows that the aerobic reactions are in general more sensitive to inhibitors than are the purely anaerobic ones. Oxygen/glucose ratios fall from about 3·8 in the control to 2·5 or less in the presence of inhibitor, indicating that under these conditions the anaerobic reaction is playing a more prominent part.

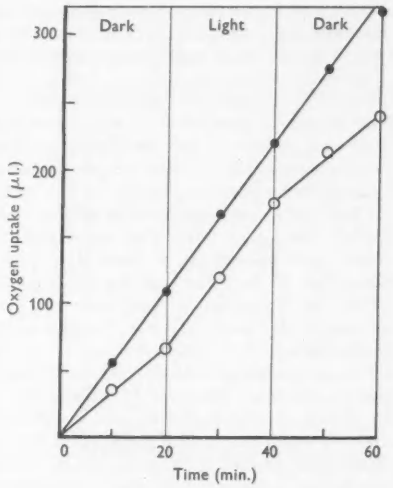

Fig. 2. Inhibition of respiration of *T. lewisi* by CO. Experiment carried out in Barcroft differential manometers at 20°; each cup contained 2·1 mg. trypanosome N and 0·02 M-glucose in 3 ml. Ringer-phosphate solution; 0·3 ml. 20% (w/v) KOH in centre well; ●——●, O_2 uptake (μl.) in presence of 5% O_2 and 95% N_2; ○——○, O_2 uptake (μl.) in presence of 5% O_2 and 95% carbon monoxide, during alternating 20 min. periods of darkness and light.

Anaerobic metabolism

Rate and pathway of anaerobic glucolysis. Values obtained of the $q^{N_2}_{CO_2}$ are rather variable. From seventeen different trypanosome preparations, values ranging from 190 to 470 were obtained, with an average of 360. The actual q value obtained depends to a large extent on the treatment of the organisms. The higher values were obtained when the trypanosomes were kept supplied with glucose during all stages in the preparation of the washed suspension; the lower ones when the flagellates were washed with glucose-free solutions and kept in such solutions during the period of manometric equilibration, prior to tipping in glucose from the side arm. Fig. 1 illustrates the progress of the anaerobic glucolysis.

Searle & Reiner (1941) state that under anaerobic conditions very little glucose is metabolized unless carbon dioxide or pyruvate is also present. The experiment described in Table 4 confirms these observations, showing that pyruvate increases glucose utilization threefold, while carbon dioxide increases it 12-fold. It is interesting to note that per mol. glucose utilized, more lactic acid is produced in the absence, than in the presence, of carbon dioxide. Table 4 also shows the relative rates of glucose utilization under aerobic and anaerobic conditions. As can be seen from the table, carbon dioxide causes only a very slight increase in glucose utilization, accompanied by a small decrease in the lactic acid yield.

Using five manometer vessels, a large-scale fermentation was carried out, to determine the end products of glucolysis. The results are given in Table 2, expressed in terms of mol. of metabolite formed per mol. glucose utilized. It can be seen from the table that these figures are fairly consistent from the point of view of carbon and acid balance. Yields of the various metabolites were variable; on some occasions, twice the yield of lactic acid has been observed, but this was accompanied by a corresponding decrease in the amount of carbon dioxide fixed and succinic acid formed. There was always a correlation between the lactic and succinic acid yields.

Table 4. *The effect of* CO_2, O_2 *and pyruvate on glucose metabolism*

(Each manometer flask contained 0·88 mg. trypanosome N, phosphate buffer pH 7·3 or 0·016 M-bicarbonate as appropriate, and 1 mg. glucose tipped in from side arm at zero time; total volume 1·6 ml. Reaction stopped after 100 min. by tipping in 0·2 ml. 2 N-H_2SO_4.)

System	Glucose used (μmol.)	Lactic acid formed (μmol.)	Ratio lactic acid/glucose
Glucose + N_2	0·39	0·57	1·45
Glucose + N_2 + 0·02 M-Pyruvate	1·36	2·08	1·52
Glucose + N_2 + 5% CO_2	4·99	2·03	0·41
Glucose + air (no CO_2)	5·10	0·85	0·17
Glucose + O_2 + 5% CO_2	5·23	0·58	0·11

Effect of various inhibitors on glucolysis. Experiments were carried out with a number of inhibitors at different levels of concentration. The inhibitor was neutralized where necessary, and made up in a solution containing 0·016 M-bicarbonate and 0·03 M-glucose. Sodium chloride was added to bring the total cationic concentration up to 0·125 M. Manometers were used containing 1 ml. trypanosome suspension in the main vessel, and 0·5 ml. of the solution containing glucose and the inhibitor in the side arm. This was tipped in after 10 min. equilibration, and the evolution of carbon dioxide was followed. Table 5 gives, in most cases, the inhibitor concentration found necessary to reduce glucolytic carbon dioxide output by 50 %. It can be seen from Table 5 that the anaerobic glucose metabolism is very sensitive to 2:2′-dipyridyl and 8-hydroxyquinoline. The effect of both these metal-binding substances develops immediately upon their addition to glycolysing organisms; in the presence of inhibitor, acid production continues at a steady, but much reduced rate. The anaerobic metabolism is also quite sensitive to iodoacetate, arsenite, cyanide and fluoride, but it is not inhibited by malonate or phenylthiourea. In view of the large anaerobic production of succinate, malonate was tested as an inhibitor; concentrations up to 0·04 M were without effect on the rate of acid production.

Table 5. *The effect of various inhibitors on anaerobic glucose metabolism*

(Manometer cups contained about 0·6 mg. trypanosome N, 0·01 M-glucose, bicarbonate buffer and 5 % CO_2–95 % N_2; total vol. 1·5 ml.; temp. 37°; pH 7·3. Glucose metabolism measured in terms of CO_2 liberated from bicarbonate.)

	Concentration (M)	Inhibition (%)
Potassium cyanide	0·007	50
Sodium azide	0·025	50
2:2′-Dipyridyl	0·0003	72
8-Hydroxyquinoline	0·0003	78
Phenylthiourea	0·001	0
Sodium fluoride	0·004	50
Sodium iodoacetate	0·0001	50
Sodium malonate	0·04	0
Phlorrhizin	0·01	50
Sodium arsenite	0·003	50

Using the concentrations of inhibitors necessary to reduce the glycolytic carbon dioxide output by about 50 %, the metabolic products of glycolysis were examined in a number of cases, to see if the yields differed in any way from the control. Manometric experiments were carried out at the same time, with and without inhibitor. The reaction was stopped by tipping in 0·4 ml. 2 N-sulphuric acid from a side bulb. After measuring the residual carbon dioxide liberated, 0·4 ml. 2 N-sodium hydroxide was added. Succinic acid was determined in a 1 ml. sample, and glucose, lactic and pyruvic acids in another 1 ml. sample, deproteinized with 10 % trichloroacetic acid. With azide or cyanide, the overall glucose utilization was reduced, but the amounts of succinic, lactic and pyruvic acids formed per mol. glucose used were not changed. With 0·005 M-fluoride, glucose utilization was reduced, and although results were rather variable, there was a definite increase in the ratios of carbon dioxide fixed and succinic acid formed, with a corresponding decrease in the yields of pyruvic and lactic acids.

The cytochrome system and cytochrome oxidase

Spectroscopic evidence. A thick trypanosome suspension was made in glucose-Ringer-phosphate solution. Examination of this suspension showed that the cytochrome was mostly in the reduced form. Reduction was completed by adding sodium dithionite ($Na_2S_2O_4$), and the suspension was examined spectroscopically at normal and at low temperatures. In the latter case, warm glycerol was added to the trypanosome suspension to give a concentration of about 50 %. A little sodium dithionite was added to reduce the cytochromes, and the mixture was introduced into a long Pyrex tube 12 mm. in diameter, the end of which had been blown to a flattened disk-shaped cell, about 25 mm. in diameter, with an optical depth of 4 mm. The tube was quickly cooled in liquid oxygen, the mixture solidifying to a transparent glass. Spectroscopic examination showed that the cytochrome bands were sharpened and displaced towards the blue, but rather weak. As the tube warmed up, the colourless vitrified mass acquired a transient amber hue, which faded as devitrification of the solid mass took place. Crystallization was accompanied by a marked intensification of the bands. The microcrystalline mass was then cooled again in liquid oxygen. Spectroscopic examination showed that the cytochrome bands were sharpened and considerably intensified, revealing a more detailed structure, and supporting the observations made at normal temperatures. At room temperature the spectrum showed:

(*a*) A definite, but very weak cytochrome *c* band at 550 mμ.

(*b*) A band extending from 553 to 560 mμ., with a definite maximum at 559 mμ., and a possible slight reinforcement at 553 mμ. This band may represent one component *e* or modified *b*, or may be due to two components, a weak *e* band at 553 mμ., and a strong *b* band at 559 mμ.

(*c*) A band at 605 mμ., representing cytochrome *a*.

(*d*) Corresponding β-bands in the region 525–535 mμ.

Manometric evidence. A homogenate of *T. lewisi* was prepared by shaking an aqueous suspension of the organisms with glass beads in the Mickle disintegrator for a few minutes. Using the system of

Keilin & Hartree (1947), it was shown that this homogenate could oxidize *p*-phenylenediamine, and that the oxidation was stimulated by added cytochrome *c* (Fig. 3). The trypanosome homogenate was also able to oxidize succinic acid, this oxidation being stimulated by added cytochrome *c* (Fig. 4). By using a small amount (0·5 mg.) of succinic acid, and allowing the oxidation to go to completion, it was shown that one atom of oxygen was used for each molecule of succinic acid oxidized. In connexion with the presence of cytochrome oxidase, the inhibitory effects of cyanide and carbon monoxide on respiration described in Table 3 and Fig. 2 should be noted.

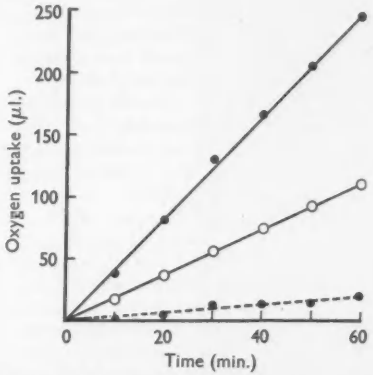

Fig. 3. Cytochrome oxidase activity in *T. lewisi* homogenate. Manometer cups contained trypanosome homogenate corresponding to about 5 mg. nitrogen, 13 mg. *p*-phenylenediamine tipped in from side arm after manometric equilibration, 0·3 ml. of 0·6% cytochrome *c* solution tipped in from second side arm, 1 ml. 0·1 M-phosphate buffer (pH 7·3), and KOH in centre well; gas phase air; temp. 37°; ●——●, O_2 uptake (μl.) of homogenate in presence of *p*-phenylenediamine and cytochrome *c*; ○——○, O_2 uptake (μl.) of homogenate in presence of *p*-phenylenediamine; ● - - - - - - ●, O_2 uptake (μl.) of *p*-phenylenediamine and cytochrome *c* in the absence of homogenate.

DISCUSSION

In rat blood, which provides the natural environment for *T. lewisi*, the survival and normal high metabolic rate of the trypanosomes depend largely on blood glucose as the source of nutrient material. A similar close dependence on glucose is shown by the trypanosomes after they have been separated from blood, and transferred into artificial, isotonic media. A general survey of the carbohydrate requirements and metabolism of trypanosomes has been recently given by von Brand (1950).

Aerobically, in absence of glucose, a suspension of washed trypanosomes in Ringer-phosphate solution, containing 10^9 protozoal cells, and representing about 9 mg. in dry weight, or about 1 mg. cellular nitrogen, takes up no more than 40 μl. oxygen/hr. at 37°. Presumably this low level of respiration is maintained at the expense of an intracellular nutrient reserve of the protozoal cells. As yet, however, there is little information on the chemical nature of this intracellular nutrient. It has been impossible to detect glycogen in the cells of *T. lewisi*, but this should not be regarded as a general characteristic of the Protozoa. In a recent, as yet unpublished study of another protozoan, *Glaucoma piriformis*, intracellular glycogen contents as high as 22% (on a dry weight basis) have been observed.

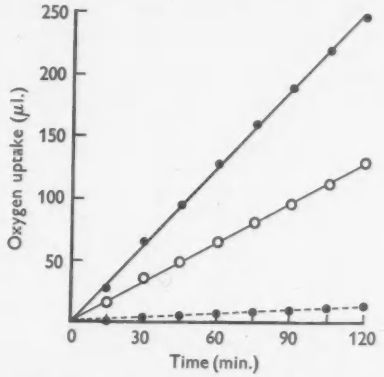

Fig. 4. Succinic oxidase activity in *T. lewisi* homogenate. Manometer cups contained trypanosome homogenate corresponding to 0·7 mg. nitrogen, in phosphate buffer (pH 7·3); 0·4 ml. of 0·2 M-succinate and 0·3 ml. of 0·6% cytochrome *c* solution tipped in from side arms after manometric equilibration; total volume 2·3 ml.; temp. 37°; gas phase air, with KOH in centre well; ●——●, O_2 uptake (μl.) of homogenate in presence of succinate and cytochrome *c*; ○——○, O_2 uptake (μl.) of homogenate in presence of succinate alone; ● - - - - - - ●, O_2 uptake (μl.) of homogenate alone.

On addition of glucose to a washed suspension of *Trypanosoma lewisi*, the rate of oxygen uptake immediately rises from 40 to 600 μl. oxygen/mg. nitrogen/hr., and remains at this high level for several hours. At the same time, the addition of glucose markedly enhances the motility of the trypanosomes. Fructose and mannose increase the oxygen uptake to the same extent as glucose, but galactose, sorbose, xylose, sucrose, maltose or lactose cause no increase. Glutamine stimulates the oxygen uptake of *T. lewisi* even more markedly than glucose, and a definite, though much smaller, increase has been observed with glycerol, asparagine and glutamic acid.

Glucose is oxidized by *T. lewisi* with an R.Q. of 0·97, and for each molecule of glucose oxidized, 3–4 mol. of oxygen are consumed, and about 1 mol.

equiv. of organic acid produced. The yield of aerobically produced acid, as revealed by the present study, is somewhat smaller than that reported by Searle & Reiner (1941), but in agreement with these authors, it was found that close to 50 % of the aerobically formed acid is derived from acetic acid. The rest is mainly composed of lactic and succinic acids. It is interesting to note that neither acetic, lactic nor succinic acids seem to be oxidized further by the intact cells themselves. This, however, does not preclude the existence in the trypanosome of intracellular enzymic systems active towards these organic acids. In cell homogenates it was possible to demonstrate the presence of lactic and succinic dehydrogenases, cozymase, and a complete succinic-oxidase system. Commenting on the fact that pyruvate, succinate, fumarate and α-ketoglutarate were not oxidized at significant rates by *T. lewisi*, Moulder (1948*a*) remarked that 'it is unlikely that the tricarboxylic acid cycle of Krebs is active in these organisms'. Analysis of the overall reaction of glucose oxidation by *T. lewisi* suggests a mechanism similar to that in the malarial parasite (Speck *et al.* 1946). With this organism, glucose is first converted to pyruvate, part of which is oxidized to acetic acid, the rest being completely oxidized by the tricarboxylic acid cycle. It was possible to demonstrate the stimulatory effects of various organic acids and co-factors only after liberation of the plasmodia from the red blood cells; it seems that the actual parasite is much more permeable to polar molecules, than is the host erythrocyte.

The present study also reveals the existence of a complete cytochrome system in *T. lewisi*. The close dependence of the respiratory activity of the trypanosome on the cytochrome system is further demonstrated by the light-reversible inhibition of the respiration by carbon monoxide. Cyanide ($4{\cdot}6 \times 10^{-4}$ M) completely blocks the respiration, and also renders the cells immotile. Dinitrophenol (10^{-3} M), on the other hand, causes almost complete inhibition of motility but only a negligible inhibition of respiration. This is presumably due to the known 'uncoupling' action of this compound on processes of oxidative phosphorylation. These results were obtained at pH 7·3, a rather high pH for the action of dinitrophenol; it is likely that the uncoupling effect of this compound would have been more pronounced at pH 6, but the trypanosomes do not respire well at this pH. Respiration is 59 % inhibited by 0·0001 M-arsenite, a value in close agreement with that found by von Brand, Tobie & Mehlman (1950). This sensitivity of respiration to arsenite is only about one-quarter of that observed with the African pathogenic trypanosomes. The effects of azide on both respiration and anaerobic glucose metabolism are interesting; usually the inhibitory effects of this substance follow closely those of cyanide. The fact that azide was found to have a much smaller effect than cyanide is probably explained by the high pH used (7·3).

Anaerobically, the trypanosomes are unable to survive unless extracellular carbohydrate is provided. In the presence of glucose under optimal conditions, the $q^{N}_{CO_2}$ may reach a figure as high as 470. For each molecule of glucose fermented anaerobically, approximately 2·5 mol. equivalents of organic acid are formed, composed mainly of succinic and lactic acids, with small amounts only of pyruvic and acetic acids. The anaerobic metabolism of *T. lewisi* was found to be extremely sensitive to a low concentration of 2:2′-dipyridyl or 8-hydroxy-quinoline, but not to phenylthiourea. From this it would seem likely that fermentation is dependent on the presence of an unco-ordinated heavy metal, quite probably ferrous iron, but not copper.

The initial stages of glucose breakdown by *T. lewisi* have not yet been elucidated. A number of trypanosomes, which are unable to ferment or oxidize glucose beyond the pyruvate stage, have been shown to carry out phosphorylative glycolysis (Chen & Geiling, 1946; Marshall, 1948; Harvey, 1949). It would seem likely, therefore, on a comparative basis, that *T. lewisi* breaks down glucose in a similar manner. The inhibitory effect of phlorrhizin on the fermentation also seems to indicate the participation of phosphorylative enzymes. However, attempts to demonstrate hexokinase activity in lysates or homogenates of *T. lewisi* have so far been unsuccessful. Direct oxidation of the glucose molecule seems a most unlikely mechanism in view of the results obtained with azide and hydroxylamine. Assuming that this type of mechanism was present, the first step in glucose breakdown would be an oxidation of glucose to gluconic acid and hydrogen peroxide, such as occurs in the acid-producing mould fungi. The peroxide would then be utilized in further oxidative reactions by the mediation of catalase. Were the catalase in such a system to be completely inhibited, glucose oxidation would lead to an accumulation of peroxide, and reduce the oxygen/glucose ratio to unity. As shown, however, in the present study (see Table 3), 0·001 M-azide or 0·0004 M-hydroxylamine, concentrations far above those necessary for total inhibition of catalase, give only small inhibitions of respiration and have very little effect on the oxygen/glucose ratio. Direct evidence of the occurrence of a phosphorylative mechanism of carbohydrate metabolism in *T. lewisi* still remains to be established.

SUMMARY

1. The motility and metabolism of *Trypanosoma lewisi* can be maintained *in vitro* both aerobically and anaerobically in the presence of suitable extracellular nutrients.

2. The respiration of *T. lewisi* depends on the cytochrome system. It is very sensitive to cyanide. It is also inhibited by carbon monoxide, this inhibition being light-reversible.

3. In the absence of extracellular nutrients, *T. lewisi* takes up oxygen at a rate of 40 μl. oxygen/mg. cellular nitrogen (or about 10^9 cells) in 1 hr. at 37°. In the presence of added glucose, mannose, fructose or glutamine, the q_{O_2} value increases from 40 to 600 or more. Glycerol, asparagine and glutamic acid increase the oxygen uptake to a smaller extent.

4. Glucose is oxidized by the trypanosome with an R.Q. of 0·97. For each molecule of glucose utilized aerobically, 3–4 mol. oxygen are taken up, and 1 mol. equivalent of organic acid is produced; half of this is acetic acid, and the rest mainly succinic and lactic acids.

5. The substances which are not utilized by the trypanosome aerobically include: galactose, sorbose, xylose, sucrose, lactose, maltose, lactate, pyruvate, succinate, citrate, α-ketoglutarate, acetate, formate, glycine, alanine and aspartic acid. Added lactate and succinate are not oxidized by intact cells, but lactic and succinic dehydrogenases could be demonstrated in cell homogenates.

6. Anaerobically, *T. lewisi* ferments glucose, the fermentation being stimulated threefold by 0·02M-pyruvate, and 12-fold by 0·016M-bicarbonate. The maximum value of the fermentation rate observed in the presence of carbon dioxide was $q_{CO_2}^{N_2} = 470$. For each mol. of glucose utilized anaerobically, 0·74 mol. carbon dioxide are fixed, and 2·5 mol. equivalents of organic acid are produced. The bulk of the acid accumulating anaerobically is succinic acid (70%) and lactic acid (15%); the rest is pyruvic and acetic acid.

7. The anaerobic fermentation is very sensitive to metal-binding substances, being inhibited by about 75% by 0·0003M-2:2′-dipyridyl and 8-hydroxy quinoline.

I should like to thank Prof. D. Keilin, F.R.S., Dr T. Mann. Dr P. Tate, and Mr C. H. Chin for much helpful advice and discussion during the course of this work, and the Medical Research Council for a maintenance grant.

REFERENCES

Biot, C., Biot, R. & Richard, G. (1911). *C.R. Soc. Biol., Paris*, **71**, 368.

Brand, T. von (1950). *J. Parasit.* **36**, 178.

Brand, T. von, Tobie, E. J. & Mehlman, B. (1950). *J. cell. comp. Physiol.* **35**, 273.

Buchanan, J. M., Sakami, W., Gurin, S. & Wilson, D. W. (1947). *J. biol. Chem.* **169**, 403.

Chen, G. & Geiling, E. M. K. (1946). *Proc. Soc. exp. Biol., N.Y.*, **63**, 486.

Feigl, F. (1937). *Spot Tests*, p. 204 Amsterdam: Nordemann Publishing Co.

Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.

Grant, W. M. (1947). *Anal. Chem.* **19**, 206.

Harvey, S. C. (1949). *J. biol. Chem.* **179**, 435.

Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.

Keilin, D. & Hartree, E. F. (1949). *Nature, Lond.*, **164**, 254.

Keilin, D. & Hartree, E. F. (1950). *Nature, Lond.*, **165**, 504.

Marshall, P. B. (1948). *Brit. J. Pharmacol.* **3**, 8.

Moulder, J. W. (1948*a*). *J. infect. Dis.* **83**, 33.

Moulder, J. W. (1948*b*). *J. infect. Dis.* **83**, 42.

Nicloux, M., le Breton, E. & Doutcheff, A. (1934). *Bull. Soc. Chim. biol., Paris*, **16**, 1314.

Reiner, L., Smythe, C. V. & Pedlow, J. T. (1936). *J. biol. Chem.* **113**, 75.

Robbie, W. A. (1946). *J. cell. comp. Physiol.* **27**, 181.

Searle, D. D. & Reiner, L. (1941). *J. biol. Chem.* **141**, 563.

Somogyi, M. (1945). *J. biol. Chem.* **160**, 61.

Speck, J. F., Moulder, J. W. & Evans, E. A. jun. (1946). *J. biol. Chem.* **164**, 119.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis: Burgess Publishing Co.

The Dimensions of the Particle of Hyaluronic Acid Complex in Synovial Fluid

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A hyaluronic acid complex was separated from ox synovial fluid by Ogston & Stanier (1950) by ultrafiltration. This complex contained about 30% protein, and was considered to be the form in which hyaluronic acid occurs in the native fluid; removal of the protein led to degradation. The authors concluded, from their results on the sedimentation and diffusion of this complex, that the mean particle weight is 1×10^6 and the axial ratio about 60. Their values for the intrinsic viscosity indicated an axial ratio of nearly 600, and they interpreted this discrepancy in terms of interaction of the particles. This interpretation is logically unsound, because all the values used had been extrapolated to zero

concentration; under this condition there could be no interaction between particles of finite size in a uniform solution.

In this paper some new measurements of viscosity and of streaming birefringence are described. It is shown that these and the former measurements are all consistent with the hypothesis that the particle weight is about 8×10^6, the axial ratio of the order of 1, that is, the particles are nearly spherical, and that the particles are very highly solvated. These properties are, in turn, consistent with the view that the particle of hyaluronic acid complex is a flexible chain.

EXPERIMENTAL

All the measurements were performed on fresh samples of ox synovial fluid, collected as described by Ogston & Stanier (1950) and dialysed against a standard buffer containing 0·2M-NaCl, 0·0077M-Na_2HPO_4 and 0·0023M-KH_2PO_4; pH 7·3.

Viscosity. The viscosities of various dilutions of synovial fluid were measured in a Couette viscometer, whose temperature was thermostatically controlled. A torsional damping device was also added, which greatly reduced oscillations of the inner cylinder and enabled more accurate measurements to be made. Viscosity was measured over a range of velocity gradient between 0·25 and 50 sec.$^{-1}$, extrapolation to velocity gradients 0 and ∞ being performed as described by Ogston & Stanier (1950). Most measurements were made at 25°, but some were also made at 37° and at 0°.

Streaming birefringence. The inner and outer (rotated) cylinders were made of vulcanite, to reduce reflexions. The diameter of the inner cylinder was 2·0 cm., the annular gap was 0·15 cm. and the length of fluid subjected to shear was 5·4 cm. The cylinders were accurately co-axial and vertical. Illumination was from a Pointolite lamp, giving a beam parallel within 0·5°. The polarizer and analyser were large Nicol prisms. The glass plate which closed the bottom of the outer cylinder and the Perspex annulus at the upper surface of the liquid were free from any detectable birefringence. A mica quarter-wave plate was used for measuring double refraction. The upper Nicol and the cross-wires (of Nylon thread) were mounted on circles divided in degrees: the angles of isocline and of extinction could be read to 0·1°.

RESULTS

Viscosity. The results were most conveniently plotted as lines of $\log \frac{1}{\phi}(\eta_{rel.} - 1)$ against volume fraction of solute, at each value of the velocity gradient (Fig. 1), where ϕ is the volume fraction of solute, assuming zero solvation. The slope of these lines falls with increasing velocity gradient. The intercept of the line for zero velocity gradient at zero concentration is the intrinsic viscosity. The remarkable feature of this set of curves is that they all make nearly the same intercept at zero concentration.

The effect of temperature on the relative viscosity is small, except at the lowest velocity gradients, where a rise of temperature causes a marked fall of relative viscosity (Fig. 2). This is illustrated also in Table 1, where the change of $\log \frac{1}{\phi}(\eta_{rel.} - 1)$ between zero and infinite velocity gradient provides a measure of the degree of viscosity anomaly. This decreases with rise of temperature, the change being almost entirely due to change of the value at zero velocity gradient.

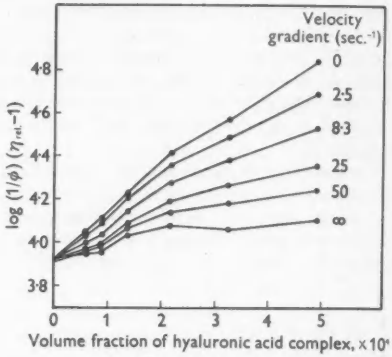

Fig. 1. Variation of $\log \frac{1}{\phi}(\eta_{rel.} - 1)$ with volume fraction ϕ at different velocity gradients.

The values for the intrinsic viscosity estimated for different samples varied between $10^{3\cdot8}$ and $10^{4\cdot0}$, these values being slightly lower than that given by Ogston & Stanier (1950).

Streaming birefringence. Measurements were made on a single (pooled) sample, which was undegraded, as judged by its viscosity. The concentration of the hyaluronic acid complex, including its protein component, was 0·082 g./100 ml. The double refraction was small, and it was not possible to measure the angle of isocline (χ) at a concentration below 0·061 g./100 ml. or the amount of double refraction ($\Delta°$) below 0·041 g./100 ml. These concentrations are high compared with those at which sedimentation and viscosity could be measured. The results are given in Table 2.

DISCUSSION

Evidence bearing on the axial ratio

Viscosity, sedimentation and birefringence. The starting-point of our present interpretation was the discovery that the value of $\log \frac{1}{\phi}(\eta_{rel.} - 1)$ at zero concentration is almost independent of the velocity gradient (Fig. 1). This can mean only that the particles, at zero concentration, are nearly spherical. It seems that the difference between the extreme

Table 1. $\log \frac{1}{\phi}(\eta_{rel.}-1)$ *as a function of velocity gradient and temperature*

(Synovial fluid 36A, $\phi = 5 \times 10^{-4}$.)

Temp. (°)	Velocity gradient G (sec.$^{-1}$) 0	25	50	∞	Change of $\log \frac{1}{\phi}(\eta_{rel.}-1)$ between $G=0$ and ∞
0·5	4·99	4·27	4·15	4·03	0·96
25	4·83	4·33	4·23	4·07	0·76
37	4·63	4·27	4·17	4·03	0·60

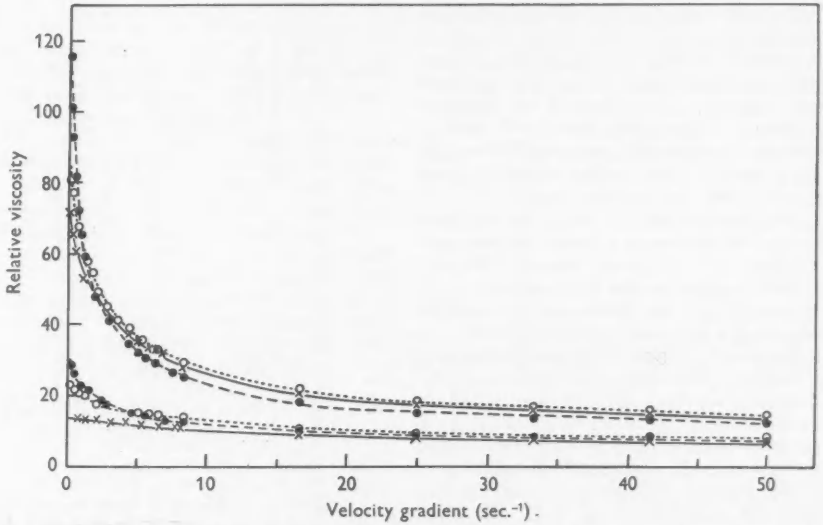

Fig. 2. Variation of relative viscosity with velocity gradient at different temperatures. – – ● – –, 0·5°; – – ○ – –, 25°; — × —, 37°. Upper set of curves, concentration = 0·08 g./100 ml.; lower set, concentration = 0·05 g./100 ml.

Table 2.

(*a*) *Values of* χ *and of* Δ *at* $G = 100$ *sec.*$^{-1}$ *for synovial fluid no.* 44 *at* 25°, *and estimates of the axial ratio* p

Concentration of hyaluronic acid complex (g./100 ml.)	$\eta_{rel.}$	$\chi°$	$\Delta°$	Estimate of p From χ	From Δ
0·082	8·0	7·5	1·9	7	22
0·061	6·5	8·1	$0·8_0$	5	18
0·041	4·2	—	$0·3_5$	—	14

(*b*) *Variation of* χ *and* Δ *with velocity gradient* G

Concentration (g./100 ml.)	G (sec.$^{-1}$)	$\chi°$	$\Delta°$
0·082	67	10·2	0·7
0·082	83	9·8	1·2
0·082	100	7·5	1·9
0·082	133	6·1	2·4
0·082	150	6·0	2·9
0·061	67	—	0·5
0·061	83	8·8	—
0·061	100	8·1	0·8
0·061	133	7·9	—
0·061	150	7·5	1·2
0·041	100	—	0·35
0·041	150	—	0·5

values of $\log \frac{1}{\phi}(\eta_{rel.}-1)$ $(\phi=0)$ cannot be greater than 0·1; then, using the theory of Mehl, Oncley & Simha (1940) for zero velocity gradient and that given by Burgers (1938, p. 144) for infinite velocity gradient, it follows that the axial ratio, assuming an elongated ellipsoid, is not greater than 3. The latter theory neglects the effect of Brownian movement, but this is unlikely to affect the conclusion that the axial ratio must be fairly small.

The very high value of the intrinsic viscosity could then be accounted for only by the assumption that the particles are very voluminous, that is, very highly solvated. It will be shown later that this is not inherently unlikely. The values of the effective specific volume V' needed to account for the intrinsic viscosity, using various assumed values of the axial ratio p and the corresponding values of ν given by Mehl *et al.* (1940), are given in Table 3.

Next, using these values of V' and p, the measured value for the sedimentation constant at zero concentration ($13{\cdot}2\times10^{-13}$, Ogston & Stanier, 1950) and Perrin's (1936) values for the frictional ratio f/f_0, values were computed for the molecular weight M and thence for the semi-major axis a (Table 3).

The corresponding expected values of the angle of isocline, were next calculated. The rotational diffusion constant θ_b was first obtained from p and a^3, using Perrin's (1934) equation. A question arose here as to which is the proper value for the viscosity to use in this calculation. Use of the viscosity of solvent gave a range of values of χ which were all larger than the measured values, while use of the viscosity of the solution measured at zero velocity gradient gave too low a range; it was therefore decided to use the measured viscosity of the solution at the concentrations and velocity gradient ($G=100$ sec.$^{-1}$) for which the computations were made. From the values of p and of $\alpha=G/\theta_b$, the values of χ were taken from the tables given by Scheraga, Edsall & Gadd (1949) (Table 3).

To calculate the expected values of the birefringence, $\Delta°$ (Foster & Edsall, 1945), the optical constant for the particles (g_1-g_2) was first computed from the equations of Peterlin & Stuart (1939). It was assumed that the particles are optically isotropic, that is, have no intrinsic birefringence; their refractive index was calculated from the measured value of the specific refractive increment of the hyaluronic acid complex (0·00153) and from V'. Δ is then given by the equations of Foster & Edsall (1945):

$$\Delta=\frac{180S}{\lambda}\frac{2\pi}{n_i}\phi'(g_1-g_2)f(\alpha,p).$$

S is the optical path length (5·4 cm.); λ the wavelength of light, taken as 546 mμ.; n_i is the refractive index of the solvent, taken as 1·33; ϕ' is the volume

Table 3. *Values calculated on the assumption of various values of the axial ratio*

Axial ratio (p)	Effective specific volume (V' ml./g.)	Molecular weight, M, $\times10^{-6}$	Semi-major axis, a, $\times10^7$ (cm.)	$a^3\times10^{15}$ (cm.^{3})	Angle of isocline $\chi°$, $G=100$ sec.$^{-1}$ (g./100 ml.)		Double refraction $\Delta°$, $G=100$ sec.$^{-1}$ (g./100 ml.)			Variation of sedimentation const. $\frac{d(1/s)}{dc}$, $\times10^{13}$
					$c=0{\cdot}082$	$c=0{\cdot}061$	$c=0{\cdot}082$	$c=0{\cdot}061$	$c=0{\cdot}041$	
1	$1{\cdot}72\times10^3$	8·8	182	6·0	—	—	—	—	—	15·6
2	$1{\cdot}48\times10^3$	8·7	273	20·3	5·7	6·8	0·017	0·015	0·010	—
3	$1{\cdot}17\times10^3$	8·5	328	35·2	—	—	—	—	—	—
5	$7{\cdot}4\times10^2$	8·1	390	58·8	7·1	8·1	0·146	0·108	0·067	6·4
10	$3{\cdot}15\times10^2$	7·2	448	89·5	7·9	8·8	0·44	0·31	0·194	2·7
20	$1{\cdot}11\times10^2$	6·3	480	110	8·5	9·3	1·34	0·97	0·58	—
50	24·3	5·2	501	125	9·1	10·1	6·1	4·4	2·6	0·04
100	7·2	4·7	512	133	9·3	10·7	19·8	14·0	8·8	—

fraction of the solute, corresponding with the weight concentration and the value of V'; $f(\alpha, p)$ is an orientation factor which has been tabulated by Scheraga *et al.* (1949). Fortunately the predicted values of Δ are rather insensitive to the value used for the viscosity.

Interpolation of the measured values of χ and of Δ in the range of calculated values (Table 3) enables the values of p to be estimated (Table 2*a*). The estimates based on Δ are the more reliable, because they are not seriously subject to the uncertainty about the viscosity, mentioned above. It should be noticed that these estimates are based on measurements at relatively high concentration; their values appear to fall with concentration, so that p might well approach 1 at zero concentration.

Diffusion and osmotic pressure. Ogston & Stanier (1950) found the diffusion constant to be about 1×10^{-7} between concentrations 0·06 and 0·3 g./100 ml. They assumed the same value at zero concentration, supposing that the variations of the entropy of dilution and of the frictional constant would be of approximately equal magnitude and opposite in sign. Experience with polymers of sarcosine (Fessler & Ogston, 1951) led us to investigate this matter more closely. The variation of the entropy of dilution is measured by the ratio

$$\left(\frac{\pi}{v_2}\right)_c \Big/ \left(\frac{\pi}{v_2}\right)_o,$$

where π is the osmotic pressure, v_2 is the volume fraction of unsolvated solute and the subscripts refer to concentration. This ratio was calculated from Huggins's (1942) equation, using a co-ordination number of 6. The calculated value at a concentration 0·184 g./100 ml. is 133. Mr O. Smithies measured the osmotic pressure of a solution of hyaluronic acid complex of this concentration and found 0·66 cm. H_2O. Assuming that the particle weight is 8×10^6, this gives a value for $\left(\frac{\pi}{v_2}\right)_c \Big/ \left(\frac{\pi}{v_2}\right)_o$ of 112, in good agreement with the calculated value.

Since $$\frac{D_c}{D_o} = \left[\left(\frac{\pi}{v_2}\right)_c \Big/ \left(\frac{\pi}{v_2}\right)_o\right] \frac{f_o}{f_c},$$

it should now be possible to calculate the variation of diffusion constant with concentration. However, a difficulty arises over the estimation of the variation of the frictional constant for the case of diffusion. Fessler & Ogston (1951) have given reasons for thinking that the diffusional frictional constant ought to vary with the viscosity of the solution, measured at zero velocity gradient, and that the variation of the sedimentation constant may not be a reliable guide to it. In Fig. 3, the predicted variations of D are shown, calculated using Huggins's theory and both the viscosity and the sedimentation constant to measure f_o/f_c. Although neither curve fits the experimental results very well, the divergence of the upper (viscosity) curve is far beyond the experimental error. Moreover, the value of D at zero concentration, obtained from the lower curve, is $0{\cdot}065 \times 10^{-7}$ and this, combined with the sedimentation constant and $\bar{V}$, gives a value for M of 16×10^6. The agreement of this with the value deduced from viscosity and sedimentation is fairly good, considering the assumptions involved.

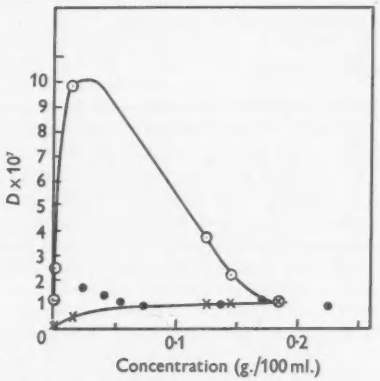

Fig. 3. Variation of the diffusion constant of hyaluronic acid complex with concentration. ●, experimental values of Ogston & Stanier (1950); ⊙, variation calculated from viscosity; ×, variation calculated from sedimentation constant.

The use of the sedimentation constant to measure the variation of the frictional constant for diffusion (rejected by Fessler & Ogston) may perhaps be justified in this case on the grounds of the extremely large size and solvated mass of the particles. This may lead to the physical process of diffusion consisting rather of the movement of solvent into a cloud of immobile particles than of the random movement of particles through solvent; this would resemble the physical process of sedimentation, where the cloud of particles, stationary relative to one another, moves through the solvent.

The variation of the sedimentation constant with concentration. Ogston & Stanier (1950) found that at low concentration $1/s$ varies linearly with the concentration; the slope $d(1/s)/dc$ (c being expressed as g./100 ml.) was $3{\cdot}90 \times 10^{13}$. Fessler & Ogston (1951) have given a theory by which this slope may be related to the values of p, V' and M; values of $d(1/s)/dc$ were calculated for different values of these quantities and are given in Table 3. The observed value is consistent only with a small value of the axial ratio.

General discussion

All the evidence cited, summarized in Table 4, points to the conclusion that the particles of the hyaluronic acid complex present in ox synovial fluid have a low axial ratio. From this it follows that the particles have an effective specific volume of the order of 10^3 ml./g. and an (unsolvated) particle weight between 8 and 9×10^6.

Table 4. *Values of the axial ratio, p*

From	Value
Variation of intrinsic viscosity with velocity gradient	< 3
Angle of isocline	< 5
Birefringence	< 14
Variation of $1/s$ with c	~ 4

The very large effective volume can be understood if the particles are of the nature of flexible chain polymers. Such particles are known to behave as if they were highly solvated because of the entrainment of solvent. The theories of Kuhn & Kuhn (1947) and of Kirkwood & Riseman (1948) predict that a random flexible chain particle would be nearly spherical. With a chain unit length of 6 A. and weight 180 and a particle weight of 9×10^6, Kirkwood & Riseman's theory predicts a mean radius of 140 mμ. which is in fair agreement with our conclusions. Synovial fluid also resembles solutions of chain polymers in showing a marked viscous-elastic behaviour (Ogston, Stanier, Toms & Strawbridge, 1950).

In view of the conclusion that the particles are nearly spherical, and of the slight variation of viscosity with velocity gradient at low concentration, the strong variation of viscosity with velocity gradient at higher concentrations requires explanation. This must presumably be due to interaction of the particles with each other; such interaction is indicated by the viscous-elastic behaviour and by the fact that the large particle volume leads to their being close together even at low values of weight concentration; for example, the volume fraction of particles reaches 1 at a concentration around 0·1 g./100 ml.

Interaction could occur by the entanglement of chains with each other, and this might lead to the linear or network aggregation of particles, both of which would increase the viscosity. Increasing the velocity gradient might have two effects, either of which would reduce the viscosity: (1) the orientation of linear aggregates parallel with the streamlines; (2) if, as is likely, the process of aggregation occupied a finite interval of time, a velocity gradient would reduce the degree of aggregation.

Since the formation of linear aggregates should markedly increase the viscosity, even at high velocity gradient (Burgers, 1938), and since the degree of aggregation must increase with concentration, the relatively small dependence of viscosity on concentration at high velocity gradient (Fig. 1) favours the second mechanism. A rise of temperature may reduce the relative viscosity by causing disaggregation; the much greater effect of temperature on the relative viscosity at low than at high velocity gradient would then also be consistent with the second mechanism, since at high velocity gradient the particles would already be largely disaggregated.

SUMMARY

1. Measurements have been made on ox synovial fluid, dialysed against buffer containing 0·2 M-sodium chloride, 0·0077 M-disodium hydrogen phosphate and 0·0023 M-potassium dihydrogen phosphate (pH 7·3) of: (*a*) the variation of viscosity with concentration, velocity gradient and temperature; (*b*) the variations of the angle of isocline and the birefringence with concentration and velocity gradient at 25°.

2. The results of these measurements are discussed in connexion with data on sedimentation and diffusion. It is concluded that all the evidence is consistent with the particles of hyaluronic acid complex being nearly spherical and very highly solvated; that the hyaluronic acid complex is probably a chain polymer; and that the variation of viscosity with velocity gradient can be explained in terms of interaction between particles.

We are most grateful to Dr J. T. Edsall for a copy of a report (Scheraga *et al.* 1949); to Dr H. Kuhn of the Clarendon Laboratory, for advice and for the loan of optical components; to Dr R. Simha for advice; to Mr O. Smithies for making a measurement of osmotic pressure; and to the Medical Research Council for a Research Grant under which this work was carried out.

REFERENCES

Burgers, J. M. (1938). *Verh. Akad. Wet. Amst.* (section *a*), **16**, no. 4.
Fessler, J. H. & Ogston, A. G. (1951). *Trans. Faraday Soc.* (In the Press.)
Foster, J. F. & Edsall, J. T. (1945). *J. Amer. chem. Soc.* **67**, 617.
Huggins, M. L. (1942). *J. phys. Chem.* **46**, 151.
Kirkwood, J. G. & Riseman, J. (1948). *J. chem. Phys.* **16**, 565.
Kuhn, W. & Kuhn, H. (1947). *Helv. chim. Acta*, **30**, 1233.
Mehl, J. W., Oncley, J. L. & Simha, R. (1940). *Science*, **92**, 132.
Ogston, A. G. & Stanier, J. E. (1950). *Biochem. J.* **46**, 364.
Ogston, A. G., Stanier, J. E., Toms, B. A. & Strawbridge, D. J. (1950). *Nature, Lond.*, **165**, 571.
Perrin, F. (1934). *J. Phys. Radium*, (7), **5**, 497.
Perrin, F. (1936). *J. Phys. Radium*, (7), **7**, 1.
Peterlin, A. & Stuart, H. A. (1939). *Z. Phys.* **112**, 1.
Scheraga, H. A., Edsall, J. T. & Gadd, J. O., jun. (1949). Report HUX-4, Physics-General, Harvard University.

A Micro-Method for the Estimation of Uronic Acids

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The method of Tracey (1948) has been modified so as to allow the estimation of the order of 0·1 mg. of free or combined uronic acid. Tracey estimated the carbon dioxide, liberated by heating the sample with hydrochloric acid in a sealed bulb, in a Van Slyke-Neill apparatus; we have used a modification of the Conway (1947) diffusion technique.

METHOD

Preparation of bulbs. The bulbs (Fig. 1, *A*) are made from 11 mm. diameter Pyrex tube. Their external diameter is about 12 mm.; they are prolonged into necks 4–5 cm. long, of diameter 3–4 mm. and drawn as thin as possible. Before use, the bulbs are washed in dilute H_3PO_4 and dried in an oven, to remove combined or adsorbed CO_2.

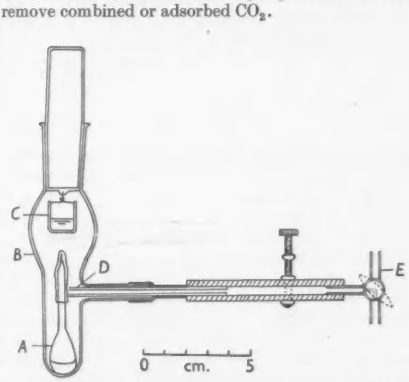

Fig. 1. Diffusion apparatus for CO_2. *A*, bulb; *B*, diffusion tube; *C*, bucket containing $Ba(OH)_2$; *D*, capillary tube; *E*, tap connecting with evacuated reservoir and soda-lime tube.

Treatment of samples with acid. The sample, containing up to 0·2 mg. of uronic acid in up to 0·4 ml., is pipetted into a bulb and 8 N-HCl is added to give a final concentration of 3·3 N. The bulb is sealed off with a small flame during evacuation on a water pump (to remove free CO_2), leaving a neck about 3 cm. long. The bulb is then heated for 5 hr. at 110–120° in an electric oven.

Diffusion of CO_2. This is carried out in a tube (Fig. 1, *B*), in which a bucket, *C*, containing $Ba(OH)_2$ can be suspended. This tube is washed with dil. H_3PO_4. The outside of the bulb is washed in dil. H_3PO_4 and dried, and a wisp of cotton wool is wrapped round its neck, to prevent glass splinters flying into the bucket. The bulb is placed in the tube, care being taken that there is no fluid in the neck. The stopper is put into the tube and a capillary tube, *D*, is fitted, with a rubber sleeve, into its side arm. The tube is then freed from CO_2 by connecting the capillary through a three-way tap, *E*, alternately to an evacuated reservoir and to an open-ended soda-lime tube. A glass bucket is filled with 0·1–0·25 ml. of approximately 0·01 N-$Ba(OH)_2$ and hung, with as little delay as possible, from the platinum hook on the stopper of the diffusion tube. The stopper is put in again, with the bucket, and the tube is at once evacuated and filled with CO_2-free air another three times. The tube is then closed, by clipping the rubber tube attached to its capillary, and the neck of the bulb is broken by pressure from the inner end of the capillary. In order to accelerate the diffusion of CO_2 into the $Ba(OH)_2$, the bottom part of each tube is placed through a hole in a circular wooden board; the board covers an open tin within which is a 40 W. lamp. The temperature of the lower part of the tube is thus maintained at 40–50° while its upper parts are cool. Under these conditions diffusion is complete in 1 hr.

Blank bulbs contain 3·3 N-HCl only; they are not heated but are introduced at the diffusion stage.

Titration. This is performed with 0·01 N-HCl, diluted by weight from constant-boiling HCl, in a 0·25 ml. Heatley (1939) microburette. Titration is done as quickly as possible, the solution in the bucket being stirred with a stream of CO_2-free nitrogen. Phenolphthalein is used as indicator. The $Ba(OH)_2$ is standardized each day. Each bucket is titrated immediately after its removal from the diffusion tube.

Miscellaneous precautions. When they are not in use, the grease used for the stoppers and all rubber connexions are kept in a desiccator over soda lime; the desiccator and its contents are evacuated from time to time.

RESULTS

The method has been tested with a sample of citrus pectin, provided by Dr M. V. Tracey, who found that it yields 12·0 g. of CO_2/100 g. Thirteen determinations gave a mean titre of 0·135 ml., with a standard deviation of 0·006 ml. (standard deviation of mean 0·002 ml.) compared with a theoretical titre of 0·136 ml. It was also tested with a sample of acetone glucurone, provided by Dr P. W. Kent; thirteen determinations gave a mean of 0·143 ml., standard deviation 0·007 ml., compared with a theoretical yield of 0·145 ml. The mean of nine determinations of the blank was 0·008 with a standard deviation 0·003.

DISCUSSION

The accuracy of this method depends on keeping the values of the blanks low, that is, on avoiding contamination with atmospheric or other carbon dioxide

as far as possible. The precautions described above have been found to be necessary in order to achieve this.

It was thought at first that a certain amount of hydrogen chloride, as well as carbon dioxide, would diffuse into the alkali. Accordingly, 10N-phosphoric acid was tried instead of hydrochloric acid, but this did not liberate all the carbon dioxide, even after heating for 24 hr. at 150°. Sulphuric and benzenesulphonic acids were also tried, but both gave high and variable yields, probably through their acting as oxidants. Sodium hydroxide was tried in the buckets instead of barium hydroxide, the carbon dioxide being estimated by the difference of titres with acid to the phenolphthalein and methyl orange end points. Finally, by use of the titrimetric method of Sendroy (1937) to estimate chloride, it was found that no detectable amount of hydrochloric acid was reaching the barium hydroxide.

Diffusion for periods greater than 2 hr. was found to be undesirable, because of a gradual increase of the blank titre. In confirmation of the work of Tracey (1948), 5 hr. heating with acid was found to liberate all the carbon dioxide from citrus pectin, acetone glucurone and from hyaluronic acid. The yield from methyl galacturonide was incomplete in this period.

SUMMARY

A modification of the method of Tracey (1948) is described, by which uronic acids may be estimated in amounts of the order of 0·1 mg. The carbon dioxide evolved by heating with hydrochloric acid is collected in barium hydroxide and estimated by titration with acid.

We are grateful to Dr F. B. Strauss for advice; to Dr M. V. Tracey for the gift of a sample of citrus pectin; to Dr P. W. Kent for samples of acetone glucurone and of methyl galacturonide; and to the Medical Research Council for a Research Grant under which this work was carried out.

REFERENCES

Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*. London: Crosby Lockwood.
Heatley, N. G. (1939). *Mikrochemie*, **26**, 147.
Sendroy, J., jun. (1937). *J. biol. Chem.* **120**, 335.
Tracey, M. V. (1948). *Biochem. J.* **43**, 185.

The Reduction of Glutathione by Plant Tissues

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The close association between glutathione (GSSG) and ascorbic acid (AA) in plant tissues is well known. In germinating seeds and sprouting potato tubers ascorbic acid and glutathione appear at the same time (Pett, 1936; Hopkins & Morgan, 1943). Moreover, many plant tissues contain the enzyme dehydroascorbic acid reductase which, as Hopkins and his collaborators have shown, catalyses the transfer of hydrogen from reduced glutathione (GSH) to dehydroascorbic acid (DHA) (Hopkins & Morgan, 1936; Crook & Hopkins, 1938; Crook, 1941).

The presence of oxidative enzyme systems catalysing the oxidation of AA, such as ascorbic oxidase, polyphenol oxidase, or peroxidase, now well established, would indicate the possibility of hydrogen transfer from GSH to oxygen via the ascorbic-dehydroascorbic acid system in accordance with the following scheme:

$$2GSH + DHA \rightarrow AA + GSSG$$
$$AA + O_2 \rightarrow DHA + H_2O.$$

How far such a system represents part of a hydrogen transfer system analogous to that of the cytochrome system depends on the ability of plant tissues to reduce GSSG. That plant tissues do possess this power is indicated by the work of Kohman & Sanborn (1937) and Ganapathy (1938), who found that compounds containing —S—S— groups could be reduced by plant juice. Even more convincing was the work which has been carried out on seeds during the initial stages of germination. Firket & Comhaire (1929) noted that, while absent in

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dry pea seeds, sulphydryl compounds rapidly appear after soaking the seeds in water. Vivario & Lecloux (1930) likewise confirmed the early appearance of sulphydryl compounds after hydration of the dry seed. Hopkins & Morgan (1943) showed that sulphydryl compounds reach a maximum within 4 hr. of adding water to the dry seed. From such material they were able to isolate reduced glutathione, and to show that if oxidized glutathione was added to pea powder suspended in phosphate buffer under anaerobic conditions the glutathione was rapidly reduced.

In the present paper an attempt has been made to identify the enzyme systems concerned. We have shown the presence in ungerminated peas of two dehydrogenase enzymes each of which is capable, in the presence of coenzyme II (Co II) and metallic catalysts such as manganese or magnesium, of transferring hydrogen from its substrate to GSSG.

The presence of an enzyme in pea seeds capable of catalysing a reaction between reduced Co II and GSSG has also been shown. In a private communication, Conn & Vennesland have informed us that they also found a similar enzyme in wheat. A preliminary account of their work and of our own has already been published (Mapson & Goddard, 1951; Conn & Vennesland, 1951).

METHODS

Acetone powder from powdered ungerminated peas. Dry ungerminated peas, variety Laxton Superb, were ground in an end-runner mill (porcelain pestle and mortar) to a powder which was sieved through a no. 40 sieve. This powder was then extracted rapidly with six times its weight of cold acetone. The acetone was removed rapidly by filtration, and the powder spread out in a thin layer on paper to remove acetone. The product, a fine powder, could be kept for a long time without loss of activity.

Dialysed enzyme. The above powder (20 g.) was mixed with 100 ml. of 0·025 M-phosphate buffer, pH 6·2, and allowed to stand 5 min. at room temperature. The mixture was centrifuged, and the supernatant liquid transferred to cellophan sacs and dialysed against 4 l. of 0·025 M-phosphate buffer at +1°. Air was bubbled through the external solution to facilitate dialysis. Dialysis was usually continued for 44–48 hr. The enzyme extract was then centrifuged to remove insoluble material precipitated during dialysis. The enzyme solution contained 0·6–0·7 g. dry solids/100 ml. Glass-distilled water was used for making the buffer solutions.

Determination of enzymic activity. The dialysed or undialysed enzyme preparation (20 ml.) was placed in a tube through which N_2 was bubbled. To this solution oxidized GSSG was added together with other substances tested and water to give a final volume of 40 ml. All experiments were done at 25°. A portion of the mixture (5 ml.) was removed at appropriate intervals and pipetted directly into 25 ml. of 4% (w/v) HPO_3, water was added to give a total volume of 50 ml. and the solution centrifuged to remove the precipitated protein. The clear supernatant was then taken for the estimation of GSH.

Oxidized glutathione. A solution of GSSG was prepared by dissolving GSH (Light and Co., Colnbrook, Bucks) in the required amount of water and oxidizing the sulphydryl group by the addition of the theoretical amount of I_2. There was no evidence of any significant impurity in the GSH used. On analysis by paper chromatography it behaved as a single substance. Solutions of GSSG were freshly prepared before each experiment. The solution was then neutralized to pH 6·3 with KOH before addition to the enzyme solution.

D-iso*Citric acid.* This was prepared from the trihydrazide of the acid, by hydrolysis with H_2SO_4 and removal of the hydrazine sulphate.

DL-*Malic acid.* This was prepared from a commercial sample recrystallized three times from water.

Coenzyme II. Two samples of Co II were used. The first sample was prepared from horse liver by a method similar to that used by Warburg, Christian & Griese (1935), for its preparation from blood. This sample was of 50% purity as determined by the increase in absorption at 340 mμ. after reduction by an *iso*citric dehydrogenase enzyme prepared from heart muscle (Grafflin & Ochoa, 1950). The second sample, prepared by the method of LePage & Mueller (1949) from horse liver, was 20% pure when determined by this method.

Reduced coenzyme II. This was made by reduction of Co II with the theoretical amount of *iso*citrate by means of an *iso*citric dehydrogenase enzyme prepared from heart muscle (Grafflin & Ochoa, 1950). After the reaction was complete the solution was brought to a pH of approx. 8·5 and heated at 100° for 2 min. to inactivate enzymes. Precipitated protein was removed by centrifugation, and the solution kept at −20° until required.

Coenzyme I. The sample used was a commercial sample of 66% purity as determined by absorption at 340 mμ. after reduction by $Na_2S_2O_4$.

Dehydroascorbic acid. This was prepared by oxidation with Br_2 according to the method described by Mapson & Ingram (1951).

Estimation of glutathione and ascorbic acid. GSH formed by reduction of GSSG was estimated by titration with 0·002 N-I_2 in acid solution. The validity of using I_2 titration as an index of the formation of GSH in these experiments was confirmed by the application of the nitroprusside test for —SH compounds. Solutions from enzyme extracts reducing I_2 gave very strong nitroprusside reactions, and conversely where the increase in I_2 titre was small or negligible, weak or negative nitroprusside tests resulted. When AA was also present, the total reducing titre of the solution was determined by titration with I_2, and the AA content by titration with 2:6-dichlorophenol indophenol in acid solution (Harris & Olliver, 1942). The titre due to GSH was then calculated by difference.

RESULTS

Reduction of glutathione by undialysed extracts of ungerminated peas

We have confirmed the work of Hopkins & Morgan (1943) that powder prepared from ungerminated peas will, under anaerobic conditions, reduce GSSG. From an acetone-extracted pea flour, an enzyme solution was prepared by treating 20 g. flour with 120 ml. 0·025 M-phosphate buffer, pH 6·3, centrifuging to remove starch and cell debris, and using the

resulting enzyme solution directly. If the oxygen in the solution is removed by nitrogen there is a rapid reduction of the disulphide when GSSG is added. The results of a typical experiment illustrated in Fig. 1 show that in the enzyme solution alone there was a steady production of titratable —SH groups which was still continuing after 24 hr. when the experiment was stopped. In the solutions to which GSSG was added at three different levels, there was at first a rapid reduction of the GSSG followed by a slower increase in titratable —SH groups at a rate equal to

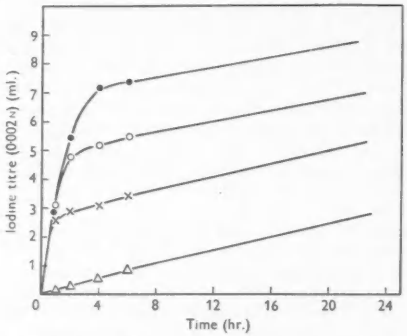

Fig. 1. Reduction of GSSG by undialysed pea extract. △—△, undialysed enzyme solution; ×—×, undialysed enzyme solution + GSSG, 0·325 mM; ○—○, undialysed enzyme solution + GSSG, 0·65 mM; ●—●, undialysed enzyme solution + GSSG, 0·975 mM. 20 ml. enzyme solution in total volume of 40 ml. of 0·0125 M-phosphate buffer, pH 6·2, in N_2. 1 ml. 0·002 N-$I_2 \equiv$ 0·615 mg. GSH.

that in the enzyme solution alone. It will be noted that while increasing the concentration of GSSG in the solution from a value of 0·325 to 0·975 mM increased the total amount of GSH formed, it did not increase the rate of the reaction. At these levels the system must be saturated with respect to GSSG. In the experiments described later we have used GSSG at a concentration of 0·65 mM, and have therefore been reasonably certain that the rate of reduction has not been limited by the concentration of GSSG. These experiments also show that addition of GSSG considerably increases the rate of production of titratable —SH groups, and suggests that in the pea, on moistening the seed, the limiting factor in the production of sulphydryl groups is the concentration of S—S compounds and not that of the enzymic activity of the tissue in transferring hydrogen or of the substrate supplying the hydrogen.

We have assumed so far that the reaction is enzymic, and the following experiment (Fig. 2) shows that extracts which had previously been heated to 100° and held at this temperature for 2–3 min. were unable to reduce GSSG and showed no increase in titratable —SH groups over a period of 20 hr. The enzymic character of the reaction is, however, more clearly demonstrated in the next experiments.

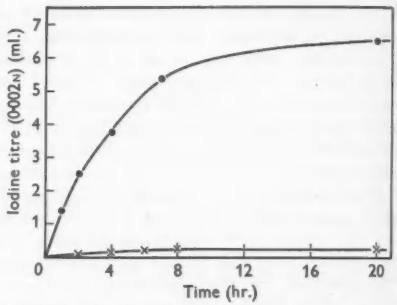

Fig. 2. Destruction of reducing activity of an undialysed pea extract by heat. ●—●, undialysed enzyme; ×—×, enzyme solution heated to 100° for 1–2 hr. GSSG in all solutions, 0·65 mM.

Reduction of glutathione in dialysed extracts of ungerminated peas

When extracts from peas were dialysed against 0·025 M-phosphate buffer, pH 6·2, at + 1° for periods varying from 17 to 72 hr. the ability of the dialysed extract to reduce GSSG was gradually lost. The

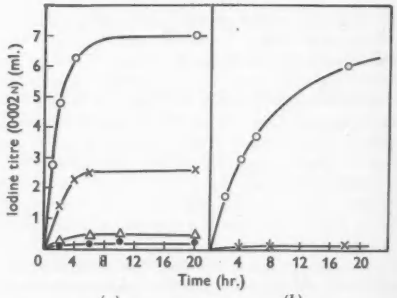

Fig. 3. (*a*) The effect of dialysis on the reduction of GSSG by an extract of dried ungerminated peas. ○—○, undialysed enzyme; ×—×, dialysed 17 hr.; △—△, dialysed 41 hr.; ●—●, dialysed 72 hr. GSSG (0·65 mM) added to each solution. (*b*) Restoration of activity to a dialysed pea extract by the addition of a boiled and filtered pea extract prepared from an acetone extracted pea powder. ×—×, extract dialysed 72 hr.; ○—○, dialysed extract + a boiled and filtered extract of pea powder. GSSG (0·65 mM) in each solution.

results of an experiment of this kind are illustrated in Fig. 3*a*. After 17 hr. dialysis, both the rate of reduction and total amount of GSSG reduced have fallen compared with the undialysed extract. After

dialysis for 41–70 hr. the enzyme extract had completely lost its power to reduce GSSG.

That this failure to reduce GSSG was not due to inactivation of an enzyme during dialysis is shown by the fact that the addition of a boiled and filtered aqueous extract from fresh acetone-extracted powder restored the ability of even the longest dialysed preparation to reduce GSSG (Fig. 3*b*).

These experiments suggested therefore that at least two factors were involved, an enzyme and a dialysable substance, presumably the substrate of the enzyme concerned. Further work was therefore carried out with the object of isolating and identifying the dialysable component.

Fractionation of acetone powder

The acetone-extracted powder prepared from the original pea meal was used as starting material. This powder was extracted with 0·0125 M-phosphate buffer (pH 6·3) for a period of 5–10 min. During this period the mixture was well stirred to facilitate extraction of water-soluble components. The powder was extracted with buffer rather than with water, for it was found that this gave a better coagulation and separation of protein material. The mixture was then centrifuged to remove starch and some insoluble protein, and the supernatant fluid was brought rapidly to the boil to inactivate enzymes and then cooled rapidly in an ice bath. This extract, when added to a dialysed enzyme preparation, restored its power of reducing GSSG.

The next stage in the fractionation consisted of adding ethanol to a concentration of 40% at room temperature. This led to a further separation of protein material without appreciably decreasing the activity of the extract. The ethanol concentration was next increased to 90% and the solution left at +1° for 4–12 hr. The whitish precipitate which separated was removed by centrifugation and redissolved in water. On testing the activity of this solution it was found to have lost about one-third of its activity. The remaining activity was found in the fraction soluble in 90% ethanol. However, despite this loss in activity, further fractionation was carried out on this fraction insoluble in 90% ethanol since considerable purification had been accomplished by this procedure.

The fraction insoluble in 90% ethanol still contained some protein material which could be precipitated by half and full saturation with $(NH_4)_2SO_4$. Tests on the protein material, however, showed it to be completely inactive. Most of the protein could be removed by adjusting the pH of the solution to 3·8, without much loss in activity in the supernatant. This solution contained some inorganic P which was removed at this stage by titration with $Ba(OH)_2$ to pH 8·3, allowing to stand at +1° for 1–2 hr. and removing the insoluble Ba salts by centrifugation. This procedure did not reduce the activity of the solution.

Further purification of the deproteinized fraction was next carried out by passing the solution through a cation-absorbing resin (Zeo-Karb 215), and the activity of the filtrate compared with that of the extract before treatment with the resin. The filtrate, when tested, showed a loss of activity, in that when added to a dialysed enzyme preparation, the rate of reduction of added GSSG was much slower, although the total amount of GSSG eventually reduced was the same (Fig. 4*a*).

This suggested the possibility that more than one component was necessary to promote full activity of the dialysed enzyme preparation, and that, besides a hydrogen donor, certain co-factors were necessary.

The fact that Zeo-Karb resin removes cations, together with the known activating effect of such ions on enzyme systems, led us to determine whether the decrease in activity of the extract, after passing through the Zeo-Karb resin, was due to this cause. The activity of this fraction was therefore re-tested with the addition of a number of inorganic salts.

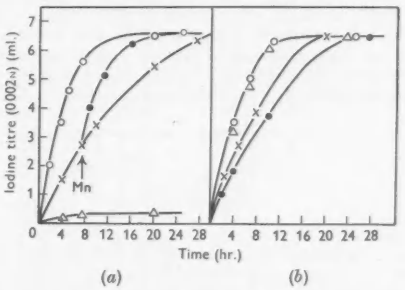

Fig. 4. (*a*) The catalytic influence of Mn on the reduction of GSSG by dialysed pea extract. (*a*) △—△, dialysed enzyme; ○—○, dialysed enzyme + substances from an aqueous extract, soluble in 40% ethanol, insoluble in 90% ethanol (90% ethanol-insoluble fraction); ×—×, dialysed enzyme + 90% ethanol-insoluble fraction after treatment with Zeo-Karb resin (Zeo-Karb fraction); ●—●, addition of Mn, $0·4 \times 10^{-3}$ M. (*b*) ●—●, dialysed enzyme + Zeo-Karb fraction; ×—×, dialysed enzyme + Zeo-Karb fraction + Mn, $0·4 \times 10^{-4}$ M; △—△, dialysed enzyme + Zeo-Karb fraction + Mn, $0·4 \times 10^{-3}$ M; ○—○, dialysed enzyme + Zeo-Karb fraction + Mn, 4×10^{-3} M. GSSG, 0·65 mM, added to all solutions.

Mn and Mg were found to be most active of the salts tried, Co and Zn salts in equivalent concentration having little catalytic effect. The results (Fig. 4) with Mn show that, under the conditions of these experiments, the minimum concentration of the metal needed to obtain the maximum effect was of the order of 0·04 mM. It seems clear, therefore, that the loss of activity of the extract on passing through the Zeo-Karb resin may have been due to the removal of metallic ions such as Mn. It will be noted that some activity with these Zeo-Karb fractions was always obtained without the addition of metallic salts, a result which may be attributed to residual traces of such metals remaining in the dialysed enzyme preparations. The difficulty of completely removing all such from enzyme preparations by prolonged dialysis is well known, and this has not yet been attempted.

Adsorption on Dowex 2 resin

The preceding experiments showed that the loss of activity on treatment with the Zeo-Karb resin was due to the removal of certain cations. The fraction was therefore further purified by passing it through a column of an anion-absorbing resin (Dowex 2). The filtrate passing this resin when tested showed no activity, indicating the absorption of the active components on the anion resin. This suggested that the active

components were probably acidic in character. However, on elution of the Dowex column with H_2SO_4, the tests on the various fractions obtained showed that the activity had been seriously reduced. This finding suggested either that the component sought had been destroyed or not eluted from the column, or that the lack of activity was due to the partial separation of two components from one another.

At this stage it seemed more profitable to examine the fractions already known to be active, to determine if it were possible to detect the presence of a hydrogen donor, which should be present in reasonable amounts, rather than attempt to separate other co-factors which might only be present in very small amounts. To do this we used the methods of paper chromatography to examine not only the fractions eluted from the Dowex column, but also the earlier fractions treated with the Zeo-Karb resin alone.

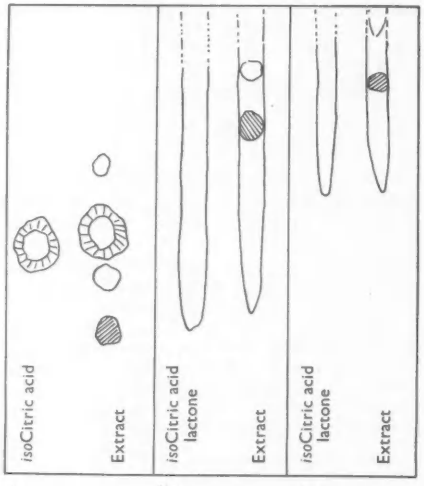

Fig. 5. Chromatograms of extracts of ungerminated pea seeds. (*a*) Chromatogram of the Zeo-Karb filtrate fraction from pea seeds. Solvent: propanol/NH_3/water (60 : 30 : 10). Spraying reagent, ammoniacal $AgNO_3$. Running time, 18 hr. *iso*Citric acid as marker. (*b*) Chromatogram of Zeo-Karb filtrate fraction after treatment with H_2SO_4 to convert *iso*citric acid to its lactone. Solvent: water-poor phase of a butanol/water/acetic acid (50 : 40 : 10) mixture. Spraying reagent, ammoniacal $AgNO_3$. Running time, 18 hr. *iso*Citric acid lactone as marker. (*c*) Chromatogram of decomposed insoluble Pb salts from Zeo-Karb filtrate fraction after treatment with H_2SO_4. Solvent and spraying reagents as in (*b*). Running time, 9 hr. *iso*Citric acid lactone as marker. Shaded areas indicate reduction of silver reagent.

Analysis by paper chromatography

With the knowledge that the unknown components in the extracts were likely to be acidic in character, we carried out an analysis to detect and identify the organic acids present. The method used was one designed by Hanes & Isherwood (unpublished) of developing a chromatogram on paper of the ammonium salts of the acids by means of a propanol/ammonia/water solvent (60 : 30 : 10). After running the acids, their position on the chromatogram can be revealed either by spraying with an alkaline solution of thymol blue or with ammoniacal silver nitrate and heating. With the first reagent the acid is indicated as a yellow spot on a blue background, and with the second either as a white or brown spot, depending on whether the acid has reducing properties.

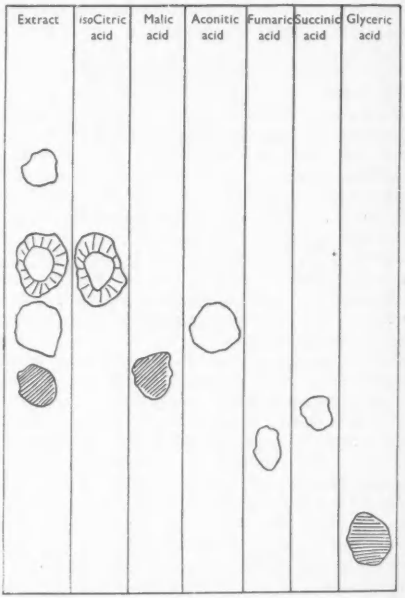

Fig. 6. Chromatogram of the decomposed insoluble Pb salts from a formic acid ethanol extract of pea seeds. Solvent: propanol/NH_3/water (60 : 30 : 10). Spraying reagent, ammoniacal $AgNO_3$. Running time, 18 hr. *iso*-Citric, malic, aconitic, fumaric, succinic and glyceric acids as markers. Shaded areas indicate reduction of silver reagent.

When the technique was applied to (1) the fraction passing the Zeo-Karb column, or to (2) the fraction eluted from the Dowex 2 resin, there was evidence of the presence of an organic acid with an R_F value suggestive of the presence of a tricarboxylic acid. This acid could also be separated as a lead salt from the extracts.

Its position on the chromatogram suggested that it was a tricarboxylic acid similar to citric or *iso*-citric acid. Further evidence suggesting it was *iso*-citric acid was obtained by treating (1) the Zeo-Karb filtrate fraction, (2) the decomposed lead salts from

this fraction, and (3) the fraction eluted from the Dowex 2 resin, with sulphuric acid and concentrating the solutions *in vacuo*. This treatment converts *iso*citric acid into its lactone. These solutions were then chromatographed on paper using as solvent the water-poor phase from a butanol/water/acetic acid (50 : 40 : 10) mixture. With this solvent the lactone of *iso*citric acid travels faster than the acid itself and leaves a characteristic trail. The following reproductions of the chromatograms obtained (Fig. 5) show in the propanol/ammonia/water solvent an acid spot on the paper similar in position to citric or *iso*citric, and after conversion to the lactone a chromatographic pattern developed in the butanol/water/acetic acid mixture similar to that given by *iso*citric acid lactone.

Chromatograms showing the presence of *iso*citric acid, together with malic and aconitic acids, were obtained by the following procedure. Ungerminated pea seeds were extracted with a solvent containing 5% (w/v) formic acid in 95% ethanol. The extract was concentrated *in vacuo* and taken up in water. After filtration neutral lead acetate was added, and the precipitated lead salts washed well with water. The lead salts were then decomposed by hydrogen sulphide and the solution neutralized with ammonia. A chromatogram of the acids was then developed using the propanol/ammonia/water solvent. A reproduction of the chromatogram is shown in Fig. 6.

Attempts to isolate these acids are being carried out and will be reported later.

Reduction of glutathione by dialysed pea enzyme with isocitrate

The foregoing data indicated the possibility that *iso*citrate was present in all of our active fractions. Experiments were therefore set up to test whether *iso*citrate could act as a hydrogen donor for the reduction of GSSG by dialysed extracts from peas. It was realized from the work of Ochoa (1945) that a coenzyme, probably Co II, as well as manganese would also be necessary. Accordingly we studied the reduction of GSSG by a dialysed enzyme preparation to which (1) *iso*citrate, (2) manganese and (3) Co I and Co II were added.

The results of an experiment of this nature are shown in Fig. 7*a*. *iso*Citrate and manganese added initially to the enzyme extract caused no reduction of GSSG. After 1·5 hr. Co I was also added without any significant result. After a further period of 2·5 hr. Co II was added. The addition of Co II caused a rapid reduction of GSSG, the reaction reaching equilibrium when most of the GSSG had been reduced. A similar experiment, illustrated in Fig. 7*b*, shows that when Co II and manganese were added initially there was no reduction of GSSG until *iso*citrate was added. It was further shown that the presence of manganese doubles the rate of the reaction. As pointed out earlier, the fact that the addition of *iso*citrate and Co II alone when added to our dialysed enzyme preparation reconstituted a system which reduced GSSG, though at a slower rate than when manganese was also added, seems to indicate that our dialysed enzyme was not completely free of metallic catalysts.

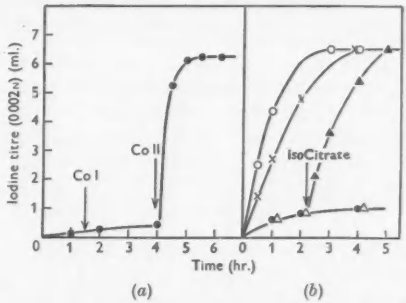

Fig. 7. The influence of *iso*citrate, Co II, Co I and Mn on the reduction of GSSG by a dialysed pea extract. (*a*) Enzyme solution contained initially D-*iso*citrate (0·005 M), Mn, $1·6 \times 10^{-3}$ M. After 15 hr. Co I (10 μg./ml.), and after a further 25 hr. Co II (5 μg./ml.) were added. (*b*) ●—●, dialysed enzyme + Co II (5 μg./ml.); △—△, dialysed enzyme + Co II + Mn ($1·6 \times 10^{-3}$ M); ▲—▲, after 2·25 hr. D-*iso*citrate added (0·005 M); ○—○, dialysed enzyme + Co II, D-*iso*citrate + Mn; ×—×, dialysed enzyme + Co II, D-*iso*citrate. GSSG, 0·65 mM, present in all solutions.

The most obvious interpretation of these facts is that the reduction of GSSG is dependent on the production of reduced Co II, this being produced by reactions similar to those described by Ochoa (1945). We may thus tentatively write the reactions involved as

(1) *iso*citrate + Co II ⇌ oxalosuccinate + reduced Co II,

$$\xrightarrow{\text{Mn}}$$

(2) oxalosuccinate ⇌ α-ketoglutarate + CO_2,

(3) reduced Co II + GSSG → Co II + 2GSH.

Table 1. *Formation of α-ketoglutarate during the reduction of glutathione*

(*System.* Dialysed enzyme + Co II (5 μg./ml.) + D-*iso*citrate, 1·25 mM + Mn, 1·6 mM + GSSG, 0·65 mM. A control solution containing all constituents with the exception of GSSG was also set up, and the small amounts of α-keto acid formed in this solution subtracted from that of the experimental solution.)

Time (hr.)	GSSG reduced (% of total added)	α-Ketoglutaric acid formed (% of theory)
0·25	12	13
1·25	52	56
3	95	106
4	97	110

Reaction (1) is catalysed by *iso*citric dehydrogenase, and reaction (2) by oxalosuccinic decarboxylase with manganese as co-factor.

Using the method of Friedemann & Haugen (1943), we have been able to show that an α-keto acid is formed during these reactions. On the assumption that the acid is α-ketoglutaric acid we have further shown that it is formed in the amounts expected on the basis of these reactions (Table 1).

Preliminary studies of the reaction between reduced Co II and GSSG are given in a later section of this paper.

Reduction of glutathione by dialysed pea enzyme with malate

The previous experiments had shown the presence in our enzyme preparations of an *iso*citric dehydrogenase, reducing Co II but not Co I. We suspected that other dehydrogenases capable of reducing Co II would, if present in our enzyme preparation, likewise catalyse the reduction of GSSG. Vennesland and her co-workers (Vennesland, Gollub & Speck, 1949; Vennesland, 1949; Conn, Vennesland & Kraemer, 1949) have shown the presence of a dehydrogenase catalysing the oxidation of malate to pyruvate in wheat germ, pea seeds, spinach and in several root vegetables. Co II is necessary and the reactions involved have been shown to be

$$\text{malate} + \text{Co II} \rightleftharpoons \text{oxaloacetate} + \text{reduced Co II},$$

$$\text{oxaloacetate} \rightleftharpoons \text{pyruvate} + CO_2.$$

(Mn)

The malate dehydrogenase was found to be Co II specific. These series of reactions are similar to those in which *iso*citrate is the hydrogen donor.

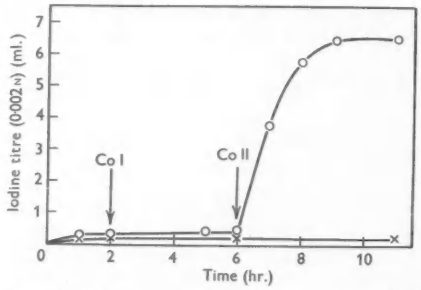

Fig. 8. The influence of Co I and Co II on the reduction of GSSG by malate. ×—×, dialysed enzyme; ○—○, dialysed enzyme + DL-malate (0·008 M) + Mn ($1·6 \times 10^{-3}$ M). After 2 hr. Co I (10 μg./ml.) added, followed by addition of Co II (5 μg./ml.) after 6 hr. GSSG, 0·65 mM, in all solutions.

Experiments were therefore set up, similar to those we have reported, using malate instead of *iso*citrate. The results illustrated in Fig. 8 show that in the presence of malate and manganese with the dialysed enzyme there is no reduction of GSSG until Co II is added. The data also show that Co I cannot serve as a coenzyme in the system.

Further experiments (Fig. 9) show (1) that Co II alone or with manganese is ineffective, in the absence of malate, and (2) that manganese acts as a co-factor. The effect of manganese in increasing the rate of the reduction was even greater than with the corresponding experiments with *iso*citrate.

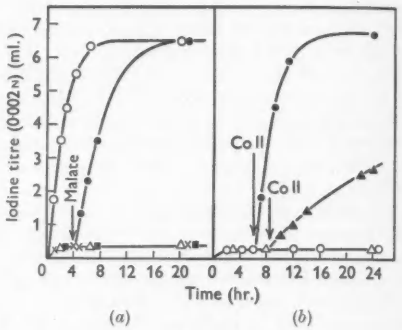

Fig. 9. Reduction of GSSG by dialysed pea extract with additions of malate, Mn, Co I and Co II. (*a*) ×—×, dialysed enzyme; △—△, dialysed enzyme + Mn ($1·6 \times 10^{-3}$ M); ■—■, dialysed enzyme + Co II (10 μg./ml.) + Mn; ●—●, after 4 hr. DL-malate (0·008 M) added; ○—○, dialysed enzyme + Co II + Mn + malate. (*b*) △—△, dialysed enzyme + DL-malate (0·008 M); ▲—▲, after 8 hr. Co II (10 μg./ml.) added; ○—○, dialysed enzyme + malate + Mn ($1·6 \times 10^{-3}$); ●—●, after 6 hr. Co II (10 μg./ml.) added. GSSG, 0·65 mM, in all solutions.

We have shown that pyruvic acid is formed during the reduction of GSSG to GSH by this enzyme system. In experiments similar to those above both GSH and pyruvic acid were estimated, the latter by

Table 2. *Formation of pyruvate during the reduction of glutathione*

(*System.* Dialysed enzyme + Co II (5 μg./ml.) + DL-malate, 2 mM + Mn, 1·6 mM + GSSG, 0·65 mM. A control solution containing all constituents with the exception of GSSG was also set up, and the small amount of pyruvic acid formed in the solution subtracted from the total pyruvic acid formed in the experimental solution.)

Time (hr.)	GSSG reduced (% of total added)	Pyruvic acid formed (% of theory)
1·5	46	42
2·23	65	59
3·5	73	72
5·0	92	87

the method of Friedemann & Haugen (1943). The amounts of pyruvic acid formed (Table 2) agreed well with those expected on the basis of the overall reaction

$$\text{Malate} + \text{GSSG} \xrightleftharpoons{\text{Co II} + \text{Mn}} 2\text{GSH} + \text{pyruvate} + CO_2.$$

The factor common to these experiments and those described for *iso*citrate is the formation of reduced Co II. It seems clear, therefore, that the essential reaction in the reduction of GSSG is

$$CoH_2II + GSSG \rightleftharpoons Co\,II + 2GSH.$$

Oxidation of reduced coenzyme II *by glutathione*

This reaction was studied by measuring the changes in absorption at 340 mμ. of reduced Co II, prepared enzymically as described in the first section of this paper, using a Hilger spectrophotometer. When GSSG was added to reduced Co II in 0·1 M-phosphate, pH 6·5, there was no oxidation of the coenzyme, and no production of GSH. When, however, the pea enzyme was added there was a rapid oxidation of reduced Co II, as determined by the decrease in absorption at 340 mμ. (Fig. 10*b*). In a similar experiment in which the pea enzyme was added to reduced Co II, there was little, if any, oxidation until GSSG was added, when a rapid oxidation of the reduced enzyme resulted (Fig. 10*a*). These experiments show clearly that the reaction reduced Co II + GSSG $\rightleftharpoons$ 2GSH + Co II is catalysed by an enzyme present in ungerminated pea seeds.

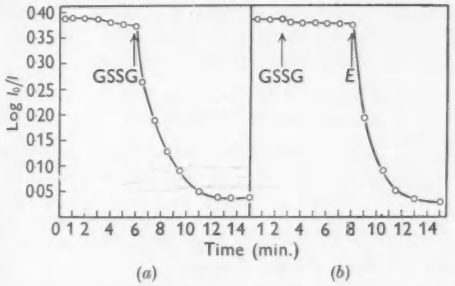

Fig. 10. The oxidation of reduced Co II by GSSG. (*a*) The system contained initially 1·5 ml. 0·1 M-phosphate buffer, pH 6·50, 0·5 ml. of dialysed pea enzyme, 130 μg. reduced Co II in a total of 3 ml. After 6 min. 250 μg. of GSSG were added. (*b*) The system contained initially 1·5 ml. 0·1 M-phosphate buffer, and 130 μg. reduced Co II in a total of 3 ml. After 3 min. 250 μg. GSSG, and after a further 5 min. the enzyme extract (*E*) were added. The reaction was followed by measuring changes in absorption at 340 mμ. in a Hilger spectrophotometer.

In further experiments it was shown that the amount of reduced Co II oxidized agreed with the equation above. Thus, in an experiment in which 0·115 μmol. of GSSG was added to a solution containing 0·17 μmol. of reduced Co II, 0·11 μmol. of Co II was produced.

We have also studied the reverse reaction, but have not been able to detect any reduction of Co II when six times the equivalent amount of GSH is added to Co II in the presence of the pea enzyme. This indicates that the equilibrium point of the reaction is very much in favour of the formation of GSH. A more detailed study of the characteristics of this enzyme is now being made, and these results will be reported later.

Experiments with coenzyme I

Bukin (1943) claimed that reduced Co I would reduce GSSG by a non-enzymic reaction, but we have not been able to confirm this. No changes in the absorption at 340 mμ. in a solution containing reduced Co I and GSSG were observed using a Hilger spectrophotometer. The Co I was reduced chemically with sodium dithionite. Nor in similar experiments could we detect any formation of reduced Co II when GSH was added to Co I.

Adler & Sreenivasaya (1937) found that pea seeds contained a formic dehydrogenase which was Co I specific. We have confirmed this, and in addition have found that the dialysed enzyme preparation contained an even more active ethanol dehydrogenase. Both enzymes reduced Co I as shown by the increase in absorption at 340 mμ. after addition of either formate or ethanol to the enzyme solution to which Co I had been added. The activities of these enzymes were measured by the Thunberg methylene-blue technique; the results of such an experiment are shown in Table 3. There was a rapid reduction of methylene blue in the presence of formate or ethanol provided Co I was also added. There was no reduction in the absence of the enzyme.

Table 3. *Reduction of methylene blue*

(Each tube contained 0·5 ml. dialysed enzyme, 1·5 ml. 0·025 M-phosphate buffer, pH 6·3, +0·5 ml. 1/10,000 methylene blue. The final concentration of coenzyme I was 100 μg./ml., of ethanol 9 mM and of sodium formate 2 mM. The final volume was 3 ml. in each tube.)

Dialysed enzyme	Sodium formate	Ethanol	Co I	Reduction time (min.)
+	+	−	+	10
+	+	−	−	>120
+	−	−	+	120
+	−	+	+	4
+	−	+	−	>120

It seems clear that the enzyme extract contained both active formic and ethanol dehydrogenases reacting with Co I. Nevertheless, when ethanol or formate was added to the enzyme preparation, together with Co I and GSSG, there was no reduction of GSSG. These experiments show, therefore, that dehydrogenases capable of reducing Co I cannot transfer hydrogen via Co I to GSSG, presumably due to lack of a suitable catalyst for the reaction between reduced Co I and GSSG.

The reduction of dehydroascorbic acid by the glutathione isocitrate *system*

We have shown that GSSG may be reduced by either the *iso*citrate or malate dehydrogenase systems present in ungerminated peas, and we felt it to be of interest to try to show the transfer of hydrogen via such systems to dehydroascorbic acid.

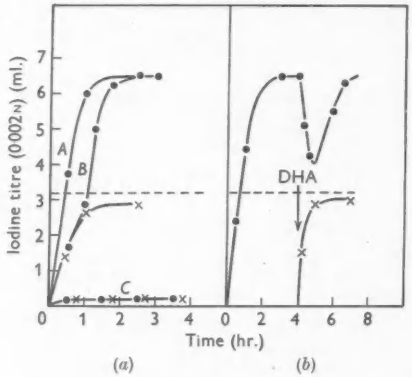

Fig. 11. The reduction of dehydroascorbic acid by dialysed pea extract. (*a*) *A*, dialysed enzyme + D-*iso*citrate (0·005 M). Mn ($1{\cdot}6 \times 10^{-3}$ M) + Co II (10 μg./ml.) and GSSG (0·65 mM). *B*, the same as *A* + dehydroascorbic acid. *C*, Dialysed enzyme + *iso*citrate + Mn + Co II + dehydroascorbic acid (0·1 mg./ml.). ●—●, GSH; ×—×, ascorbic acid. (*b*) Dialysed enzyme + *iso*citrate + Co II + Mn + GSSG. After 4 hr. dehydroascorbic acid (0·1 mg./ml.) added. In both graphs the dotted line indicates the amount of dehydroascorbic acid added. ●—●, GSH; ×—×, ascorbic acid.

A dialysed enzyme preparation was set up with *iso*citrate, Co II, manganese and GSSG, and the reduction of GSSG followed as in previous experiments. To one tube no further addition was made; to the other a known amount of dehydroascorbic acid. Estimations of GSH in the first case and of GSH and ascorbic acid in the second case were carried out. The results (Fig. 11*a*) show that, in the absence of dehydroascorbic acid, a rapid formation of GSH occurs. In the presence of dehydroascorbic acid the accumulation of GSH occurs more slowly. When the reduction of dehydroascorbic acid is complete, the rate of accumulation of GSH increases and continues until it reaches the same value as that reached in the control solution. In the absence of GSSG there was no reduction of dehydroascorbic acid. These observations are the result of two opposing reactions: the reduction of GSSG to GSH, which proceeds at the same rate in the solution to which dehydroascorbic acid is added as in the control, and the oxidation of GSH by dehydroascorbic acid, which reduces the net accumulation of GSH. This is borne out by the curves (Fig. 11*a*) where, in the initial stages of the reaction, the sum of GSH and ascorbic acid produced is very nearly equal to the amount of GSH produced in the control experiment.

In a following experiment (Fig. 11*b*) the reduction of dehydroascorbic acid at the expense of GSH is again clearly shown. In this experiment the dialysed enzyme with Co II, *iso*citrate, manganese and GSSG was allowed to reach equilibrium. At this stage dehydroascorbic acid was added. There was a rapid reduction of the dehydroascorbic acid which was correlated with a fall in the GSH content. After the reduction of the dehydroascorbic acid, there was again a rise in GSH until the original value was reached.

These experiments demonstrate the transfer of hydrogen from *iso*citrate to dehydroascorbic acid in accordance with the following scheme:

$$\textit{iso}\text{citrate} + \text{GSSG} \xrightarrow{\text{Co II} + \text{Mn}} 2\text{GSH} + \alpha\text{-ketoglutarate} + \text{CO}_2$$

$$2\text{GSH} + \text{DHA} \rightarrow \text{GSSG} + \text{AA}.$$

This system, in combination with ascorbic oxidase or similar enzymes, will therefore effectively transfer hydrogen from substances such as *iso*citrate or malate, and since both are intermediates in the Krebs cycle, we have the possibility of a link here with the normal respiratory mechanisms.

DISCUSSION

Mann (1932) observed the reduction of GSSG by glucose and glucose dehydrogenase prepared from liver. He found, however, that the rate of this reaction was slow, but could be accelerated by an activator extracted from liver tissue. The nature of this activator was not disclosed.

Meldrum & Tarr (1935) observed that GSSG was reduced by enzyme extracts from blood or yeast in the presence of hexosemonophosphate and extracts containing Co II.

The work presented in this paper has shown a hydrogen transfer mechanism, whereby hydrogen from certain intermediates, derived ultimately from carbohydrate, may pass to molecular oxygen via Co II, GSSG and dehydroascorbic acid. In this respect the system provides an alternative respiratory pathway to that of cytochrome and links both GSH and ascorbic acid with Co II, which itself is one central point in the hydrogen-activating mechanisms of the cell.

We have at present no direct information how important such a system is in the general respiratory activity of the pea seed, or how active it is, in relation to that of cytochrome. As far as our knowledge goes, this enzyme system, like that of cytochrome, would be cyanide sensitive, for all the terminal

oxidases, i.e. ascorbic oxidase, polyphenolase, and peroxidase known to oxidize AA in plant tissues, are poisoned by cyanide.

The evidence that a close correlation exists between GSH and AA in plant tissues has already been summarized in the introduction. Direct evidence that there is a connexion between the state of oxidation of GSH and that of AA has recently been shown by Barker & Mapson (1951), who found that when potato tubers are subjected to an atmosphere of pure oxygen there is first a fall in the GSH content of the tissue, followed closely by a fall in AA and a rise in DHA. This evidence, although incomplete, suggests that the AA content of the tissue is dependent on the maintenance of its GSH content.

We have no indication yet of the nature of the enzyme catalysing the reaction between reduced Co II and GSSG, which by analogy with cytochrome reductase we might term glutathione reductase. From our knowledge of the nature of diaphorase and cytochrome reductase, all we may do at this stage is to speculate whether this enzyme too may be a flavoprotein.

SUMMARY

1. Extracts from ungerminated pea seeds under anaerobic conditions rapidly reduce glutathione (GSSG) to reduced glutathione (GSH). When these extracts are dialysed there is a progressive fall in the activity of the extract.

2. There is chromatographic evidence that both *iso*citric and malic acids are present in pea seeds.

3. The activity of these dialysed extracts may be restored by the addition of *iso*citrate or malate, coenzyme II (Co II) and manganese. With *iso*citrate, α-ketoglutaric acid and with malate, pyruvic acid is formed in amounts equivalent to the glutathione reduced. Coenzyme II cannot be replaced by coenzyme I.

4. Reduced coenzyme II does not reduce glutathione in solution in the absence of enzymic catalysts. Dialysed pea extracts contain an enzyme which actively catalyses the reaction

$$\text{Reduced Co II} + \text{GSSG} \rightleftharpoons \text{Co II} + 2\text{GSH}.$$

The reduction of glutathione is equivalent to the amount of coenzyme II oxidized.

5. The reverse reaction between coenzyme II and reduced glutathione could not be demonstrated, indicating that the equilibrium is in favour of the formation of reduced glutathione.

6. The presence in the dialysed pea extracts of ethanol and formic dehydrogenase enzymes, both coenzyme I specific, has been shown. These enzymes in the presence of their substrates and coenzyme I will not reduce glutathione; reduced coenzyme I will not reduce glutathione, nor was any evidence obtained of the presence of an enzyme which would catalyse the reaction.

7. Dehydroascorbic acid is rapidly reduced to ascorbic acid when it is added to a dialysed enzyme extract containing isocitrate, coenzyme II, and manganese glutathione. The reduction of dehydroascorbic acid is dependent on the production of reduced glutathione.

8. When dehydroascorbic acid reductase is present, a system is complete by which hydrogen may be transferred in turn from the hydrogen of substrates of enzymes reducing coenzyme II to glutathione and dehydroascorbic acid.

We wish to thank Dr S. M. Partridge and Mr R. C. Brimley for their participation in that part of the work in which ion-exchange resins were used. We also wish to thank Dr J. Barker for his interest and advice.

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REFERENCES

Adler, E. & Sreenivasaya, M. (1937). *Hoppe-Seyl. Z.* **249**, 24.
Barker, J. & Mapson, L. W. (1951). *New Phytol.* (in the Press).
Bukin, V. N. (1943). *Biochimia*, **18**, 60.
Conn, E. & Vennesland, B. (1951). *Nature, Lond.*, **167**, 976.
Conn, E., Vennesland, B. & Kraemer, L. M. (1949). *Arch. Biochem.* **23**, 179.
Crook, E. M. (1941). *Biochem. J.* **35**, 226.
Crook, E. M. & Hopkins, F. G. (1938). *Biochem. J.* **32**, 1356.
Firket, M. I. & Comhaire, Mlle. (1929). *Bull. Acad. Méd. Belg.* Series 5, **9**, 93.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Ganapathy, C. V. (1938). *Curr. Sci.* **6**, 451.
Grafflin, A. L. & Ochoa, S. (1950). *Biochim. Biophys. Acta*, **4**, 205.
Harris, J. & Olliver M. (1942). *Biochem. J.* **36**, 155.
Hopkins, F. G. & Morgan, E. J. (1936). *Biochem. J.* **30**, 1446.
Hopkins, F. G. & Morgan, E. J. (1943). *Nature, Lond.*, **152**, 288.
Kohman, E. F. & Sanborn, N. H. (1937). *Industr. Engng Chem.* **20**, 189, 1195.
LePage, G. A. & Mueller, G. C. (1949). *J. biol. Chem.* **180**, 975.
Mann, P. J. G. (1932). *Biochem. J.* **26**, 785.
Mapson, L. W. & Goddard, D. (1951). *Nature, Lond.*, **167**, 975.
Mapson, L. W. & Ingram, M. (1951). *Biochem. J.* **48**, 551.
Meldrum, N. V. & Tarr, L. H. A. (1935). *Biochem. J.* **29**, 108.
Ochoa, S. (1945). *J. biol. Chem.* **159**, 243.
Pett, L. B. (1936). *Biochem. J.* **30**, 1228.
Vennesland, B. (1949). *J. biol. Chem.* **178**, 591.
Vennesland, B., Gollub, M. C. & Speck, J. F. (1949). *J. biol. Chem.* **178**, 301.
Vivario, R. & Lecloux, J. (1930). *Arch. int. Physiol.* **32**, 1.
Warburg, O., Christian, W. & Griese, A. (1935). *Biochem. Z.* **282**, 157.

Studies on the Enzymic Oxidation of Succinic Acid Containing Deuterium in the Methylene Groups

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The assumption on which all metabolic experiments involving tracer isotopes is based is that atoms, groups of atoms, and molecules containing the tracer isotope react in a manner identical with those of normal constitution. However, the theory and practice of the concentration and estimation of stable isotopes shows that discrimination between the isotopes of an element is possible. The question therefore arises as to what extent the living organism provides conditions for discrimination between isotopes. The work to be described here is concerned with differences in behaviour of a normal substrate and the corresponding deuterated substrate in an enzyme reaction.

Differences in behaviour of isotopic compounds are most likely to occur in the case of hydrogen and deuterium compounds because the tracer element is approximately double the mass of the normal element. A normal compound and the corresponding deuterated compound may not be expected to react at the same rate if the difference in mass of hydrogen and deuterium causes a sufficiently marked difference between the zero-point energies (and hence the activation energies for a given reaction) of the two compounds. Indeed, theoretical calculations by Eyring & Sherman (1933) indicated that in chemical reactions at ordinary temperatures, deuterium would be expected to react at least 50% more slowly than hydrogen. These calculations have been supported by results of investigations of relative rates of proton and deuteron transfer (e.g. Wynne-Jones, 1934; Wilson, 1936; Bonhoeffer, 1938). In the biological field, much of the earlier work was devoted to experiments on the effects of heavy water (D_2O) on biological systems. The dramatic demonstration by Lewis (1933) of the failure of tobacco seeds to germinate in pure D_2O was followed by a large volume of work which broadly suggested that low concentrations of D_2O (up to about 10%) had no influence on biological systems (e.g. Dungern, 1936), while higher concentrations in general retarded to varying degrees a number of biological processes, for example, the respiration and alcoholic fermentation of yeast (Pacsu, 1934; Taylor & Harvey, 1934; Hughes, Yudkin, Kemp & Rideal, 1934) and the metabolic rate of mice (Barbour, 1934). Further work was carried out in an attempt to determine whether any alteration of the deuterium/hydrogen ratio occurred in biological systems. This work resolved itself into the careful comparison of the densities of waters of biological origin with the density of a standard natural water. Since the determinations were carried out by density methods, it is not certain, in many cases, to what extent the results might have been affected by variations in the ratio of the oxygen isotopes. The subject of the deuterium/hydrogen ratio constitutes a field on its own (see, for example, Emeleus, James, King, Pearson, Purcell & Briscoe, 1934; Dole, 1936) and cannot be discussed here in detail. It may be concluded from the results, however, that concentration of neither of the isotopes of hydrogen occurs to any great extent in biological systems. On the other hand, the results do not exclude the possibility that small variations in the amount of deuterium may occur in biological systems (say up to about 3% of that normally present), since a variation of this magnitude would be within the limits of error of the several density determinations necessary to control interference from variations in the ratio of the oxygen isotopes (Dole, 1936). In summary it can be said that although on theoretical grounds hydrogen and deuterium may be expected to differ in reactivity, no effect of low concentrations of D_2O on biological systems has been shown satisfactorily, nor has there been a convincing demonstration of biological enrichment of a hydrogen isotope. Such experimental results as these have been taken as the justification for a great deal of tracer work which has been carried out with deuterium.

In discussing the applicability of deuterium as a biological tracer for hydrogen, it is necessary to distinguish between the comparison of the rates at which a compound reacts in media of normal water and D_2O, and the comparison of the rates at which proton and deuteron transfer reactions take place. For example, if a reaction, which involves protons or deuterons, is found to proceed at the same rate in dilute D_2O as in normal water, it is not justifiable to conclude that the deuterium/hydrogen ratio in the reaction products is the same as the deuterium/hydrogen ratio in the medium. And although dilute

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solutions of D_2O have no apparent effect on many biological processes, it cannot be assumed that deuterium follows the reactions of hydrogen at the same rate, nor does it follow that an enzyme will interact with a deuterated substrate in a manner identical with its normal substrate. The biological work referred to above was concerned with dilute D_2O as a reaction medium; comparatively little appears to have been done in the biochemical field on proton and deuteron transfer.

In order to assist in clarifying the position it seemed desirable to compare the action of a dehydrogenase on its normal substrate with its action on the deuterium-substituted substrate. The succinic oxidase system, although complicated and not fully understood, appeared to be the most convenient for such a study since the methylene hydrogen atoms of succinate are not immediately exchangeable with the hydrogen ions of the aqueous medium. Using deuterium as a tracer, however, it has been shown that considerable exchange of succinic acid methylene hydrogen atoms with hydrogen ions takes place when a solution of succinate is maintained at 80° for 100 hr. (Wynne-Jones, 1935) or is incubated anaerobically with a crude succinoxidase preparation (Weinmann, Morehouse & Winzler, 1947). It was therefore evidently necessary to use as active as possible a preparation of the succinic oxidase system, in which the rate of reaction is not limited by an insufficient concentration of intermediate carriers, and to obtain information from experiments of short duration.

Erlenmeyer, Schonauer & Süllmann (1936) found that [$\alpha\alpha'$ D_2] succinic acid (dideuterosuccinic acid) was oxidized at about 70% of the rate of oxidation of normal succinic acid in the presence of a crude muscle mince and methylene blue. It was proposed to extend this aspect of their experiments by studying the kinetics of the reaction in greater detail. Since the acid which they used contained two hydrogen and two deuterium atoms in the methylene groups there appeared to be an uncontrollable factor involved as to which two of these four atoms were removed, and therefore it is not possible to compare rates of proton and deuteron transfer using this acid as a deuterated substrate. For this reason the action of the succinic oxidase system on normal succinic acid was compared with its action on [$\alpha\alpha\alpha'\alpha'$ D_4] succinic acid (tetradeuterosuccinic acid) in order to compare rates of proton and deuteron transfer. The reactions of the two substrates were further compared with respect to activation energy, and an estimate of the relative affinity of the enzyme for the two substrates was also made.

This work forms part of a thesis presented for the Degree of Ph.D. in the University of London. A preliminary account has been given to the Biochemical Society (Thorn, 1949*a*).

MATERIALS AND METHODS

[$\alpha\alpha\alpha'\alpha'D_4$] *Succinic acid* was prepared by the reduction of potassium acetylenedicarboxylate with sodium amalgam in D_2O, substantially as described by Halford & Anderson (1936). The potassium acetylenedicarboxylate was obtained by the reaction of dibromosuccinic acid with KOH in ethanol (Moureu & Bongrand, 1920) and was not separated from the KBr which was simultaneously produced, since the latter does not interfere with the reduction. For the reduction, 8·0 g. of the salt mixture were partially dissolved in 10 ml. of D_2O (99·77%), and, while the mixture was cooled in an ice bath and mechanically stirred, an amalgam made from 2·0 g. of sodium and 20 ml. of mercury was added to it over a period of 40 min.

The purification methods of Halford & Anderson were not followed because they were designed to produce an acid containing deuterium also in the carboxyl groups. The D_2O was first recovered from the reaction mixture by vacuum distillation. The resulting mass of crystals was dissolved in 60 ml. of water, and the solution was acidified to congo red, transferred to an extractor with washing to 150 ml., and continuously extracted with ether for 8·5 hr. The crude tetradeuterosuccinic acid, obtained by evaporating the ether extract to dryness, was redissolved in 100 ml. of 0·75 N-H_2SO_4 and treated with 0·75 N-$KMnO_4$ at boiling point. This permanganate treatment, which removes small amounts of incompletely reduced material, is in principle the same as that used in similar circumstances by Weinmann *et al.* (1947), following the method of Goepfert (1940). The purified tetradeuterosuccinic acid was recovered from this solution by continuous ether extraction for 6·5 hr. After evaporation of the ether, the yield of the acid was 70% (based on the weight of the salt mixture used for reduction). The acid recrystallized from water had m.p. (uncorr.) 181·8–182·6°.

The acid was found to contain 77 atom % D in the methylene groups instead of the theoretical 100 atom %. The reason for the low result was not sought, but deuterium may have been lost by exchange during the purification and recrystallization.

[$\alpha\alpha'D_2$] *Succinic acid* was prepared by reduction of recrystallized fumaric acid by methods similar to those described above. Since the reaction mixture contained no KBr it was treated with acid permanganate immediately after the recovery of D_2O, and was then continuously extracted with ether. The yield was 72%. The extracted acid, recrystallized from water, had m.p. (uncorr.) 182·7–183·4°.

The acid was found to contain 42 atom % D in the methylene groups instead of the theoretical 50 atom %.

Normal succinic acid was prepared during trials of the methods described, using water in place of D_2O as the medium for sodium amalgam reduction. The acid had m.p. (uncorr.) 182·8–183·6°. (Found: C, 41·2; H, 5·2. Calc. for $C_4H_6O_4$: C, 40·7; H, 5·1%.) Analysis by Weiler & Strauss, Oxford.

This acid was used for comparison with the deuterated acids in the enzyme experiments.

Deuterium analysis. The deuterated acids were analysed for deuterium by the gradient tube method (Linderstrøm-Lang & Lanz, 1938; Linderstrøm-Lang, Jacobsen & Johansen, 1938; Anfinsen, 1946). The combustion of the samples was carried out substantially as described by Keston, Rittenberg & Schoenheimer (1937) and the samples of combustion water were purified in a distillation apparatus similar to that

described by these workers. The water samples were refluxed in the apparatus for a few minutes over $KMnO_4$ and CaO, and were then distilled *in vacuo* three times.

Succinic oxidase system. The system described by Keilin & Hartree (1940) was used. The preparation of the enzyme system (Keilin & Hartree, 1938) differed from that of the original authors in that the washed mince of pig-heart muscle was broken down with phosphate buffer in a Waring blender, instead of in a mechanical mortar with sand. Cytochrome *c* was prepared according to Keilin & Hartree (1937).

Measurement of oxygen consumption. The rate of oxygen consumption of the system was measured in Warburg manometers at 37–37·5° (unless otherwise stated). The gas phase was air. In the several series of experiments which were carried out the concentrations of the constituents of the mixtures in the flasks varied as follows: phosphate buffer, pH 7·3, 0·04–0·05 M; succinate (the acids were neutralized to pH 7·3 with NaOH before making their solutions up to volume), 0·02–0·05 M; cytochrome *c*, $0{\cdot}76 \times 10^{-5}$–$1{\cdot}4 \times 10^{-5}$ M. The manometers were read at 5 min. intervals for about 30 min., during which period the reaction velocity was sensibly constant. The oxygen consumption was plotted against time, and from the best straight line (judged by eye) the oxygen consumption at 15 min. (unless otherwise stated) was taken as a measure of reaction velocity.

The substrate was usually added from the side arm after equilibration in order to avoid contact of the deuterated substrate with the enzyme before readings were commenced. Preliminary experiments were undertaken in order to determine concentrations of substrate and cytochrome *c* above which the reaction velocity was not increased.

RESULTS

Rates of oxidation of succinic acid, tetradeuterosuccinic acid and dideuterosuccinic acid in the presence of the succinic oxidase system. The rate of oxygen consumption of the succinic oxidase system in the presence of tetradeuterosuccinic acid was expressed as a percentage of that in the presence of succinic acid. The mean result from twelve experiments was 39·0 % (range, 34·1–42·7 %). That is to say, tetradeuterosuccinic acid was oxidized at about 40 % of the rate of oxidation of succinic acid. A typical experiment is illustrated in Fig. 1.

In the same manner, it was found from four experiments that dideuterosuccinic acid was oxidized at about 70 % of the rate of oxidation of succinic acid (mean, 71·2 %; range, 65·5–81·4 %). This value is in agreement with the results of Erlenmeyer *et al.* (1936).

It must be remembered that these results, and all others given in this paper, refer to deuterated acids containing about 20 % less deuterium in the methylene groups than is implied by their names. It is to be expected, therefore, that tetradeuterosuccinic acid and dideuterosuccinic acid would be oxidized by the succinic oxidase system at rates (relative to the rate of oxidation of succinic acid) even less than those recorded here.

Activation energies of the oxidation of succinic acid and tetradeuterosuccinic acid in the presence of the succinic oxidase system. Two experiments were carried out in which the influence of temperature on the rates of oxidation of succinic acid and tetradeuterosuccinic acid in the presence of the succinic oxidase system was determined. In the first experiment the rates were determined at 20·2, 28·0 and 37·0°, and in the second at 20·2 and 37·0° only. Duplicate determinations were carried out in all cases. Owing to slight daily variations in the activity of the enzyme each experiment was completed in 1 day. The logarithms of the rates were plotted against the reciprocals of the absolute temperatures as shown in Fig. 2, and by multiplying the negative slopes by $2{\cdot}303R$, the values for the activation energies were obtained, as shown in Table 1.

Table 1. *Activation energies of the oxidation of succinic acid and tetradeuterosuccinic acid in the presence of the succinic oxidase system*

Exp. no.	Activation energy (cal.) Succinic acid	Tetradeuterosuccinic acid
22	11,100	12,400
23	11,400	13,000
Average	**11,250 ± 150***	**12,700 ± 300**

* The value of 11,500 cal., previously reported (Thorn, 1949*a*), was incorrect owing to an error in calculation.

The results indicate that there is a difference between the activation energies of the reactions of the two substrates of 1450 ± 450 cal., the slower reaction of the deuterated substrate being associated with the higher activation energy. The value obtained for the activation energy of the reaction of succinic acid is in agreement with that obtained by Hadidian & Hoagland (1939).

The Michaelis constants of the succinic oxidase system with respect to succinic acid and tetradeuterosuccinic acid. The influence of substrate concentration on the rate of oxygen consumption of the succinic oxidase system was determined in the case of succinic acid and tetradeuterosuccinic acid, and from the results the Michaelis constants of the system, with respect to succinic acid and tetradeuterosuccinic acid, were calculated. Owing to the fact that at low substrate concentrations the reaction velocity falls very soon after the reaction has begun, it was necessary to read the manometers at 3 min. or even 2 min. intervals in order to obtain as reliable an estimate as possible of the initial reaction velocity. It was then possible to obtain at least two readings that lay on a straight line, as shown in Fig. 3. The reaction velocity was taken as the oxygen consumption at 6 min., read from the straight line. A number of experiments was performed, out of which three on each substrate, from which reasonable estimates of initial velocities could be obtained,

were used for calculating Michaelis constants. The Michaelis constants were calculated from the data by plotting s/v against s, where s is the substrate concentration and v the initial reaction velocity, according to the method of Lineweaver & Burk (1934). The slopes and intercepts of the lines (one of which is shown in Fig. 4) were calculated by the

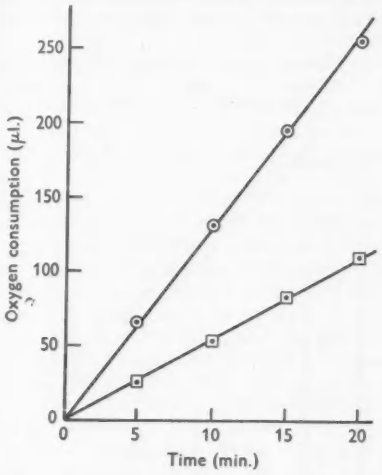

Fig. 1. Oxygen consumption of the succinic oxidase system: ⊙, in the presence of succinate; ⊡, in the presence of tetradeuterosuccinate.

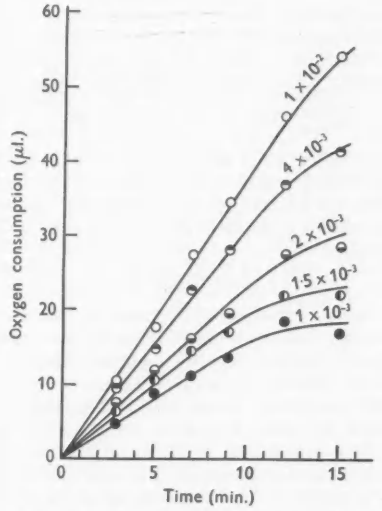

Fig. 3. The effect of succinate concentration (M) on the oxygen consumption of the succinic oxidase system (Exp. 12).

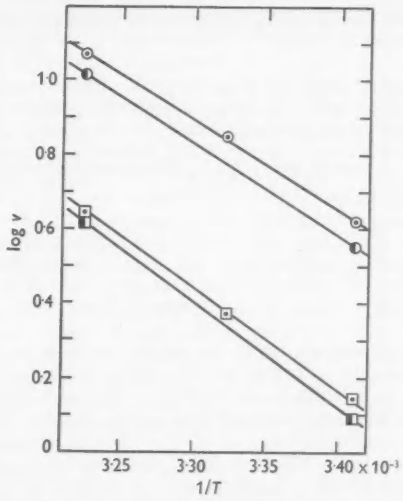

Fig. 2. The effect of temperature on the rate of oxygen consumption of the succinic oxidase system in the presence of succinate and tetradeuterosuccinate. v = rate of oxygen consumption (μl./min.); T = absolute temperature. ⊙, succinate, Exp. 22; ◐, succinate, Exp. 23; ⊡, tetradeuterosuccinate, Exp. 22; ◧, tetradeuterosuccinate, Exp. 23.

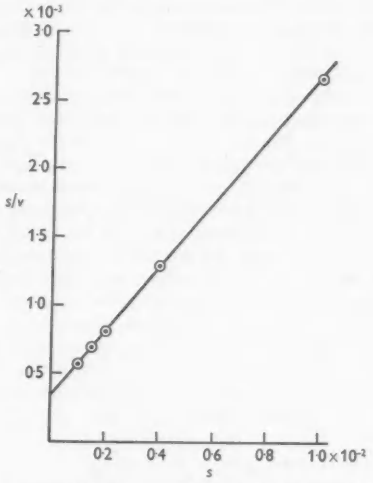

Fig. 4. The effect of succinate concentration on the rate of oxygen consumption of the succinic oxidase system (Exp. 12), plotted according to Lineweaver & Burk (1934). s = molar concentration of succinate; v = initial rate of oxygen consumption (μl./min.).

method of least squares, and from them were obtained the values for the Michaelis constants shown in Table 2.

Table 2. *Michaelis constants of the succinic oxidase system with respect to succinic acid and tetradeuterosuccinic acid*

Succinic acid		Tetradeuterosuccinic acid	
Exp. no.	K_m (M)	Exp. no.	K_m (M)
10	$0{\cdot}86\times10^{-3}$	7	$2{\cdot}06\times10^{-3}$
11	$1{\cdot}27\times10^{-3}$	8	$2{\cdot}63\times10^{-3}$
12	$1{\cdot}53\times10^{-3}$	9	$1{\cdot}77\times10^{-3}$
Average	$1{\cdot}2\times10^{-3}$	Average	$2{\cdot}2\times10^{-3}$

The average figure for succinic acid is in fair agreement with that of $1{\cdot}0\times10^{-3}$ M calculated by Haldane (1930) from the data of Widmark (1922). The results for each acid do not agree closely, which is not surprising in view of the difficulty of making reliable estimates of the initial reaction velocities at low substrate concentrations. Nevertheless, there appears to be a difference between the two sets of figures, indicating a value for the ratio K_m^D/K_m^H of about 1·8, where the symbols refer to the Michaelis constants of tetradeuterosuccinic acid and succinic acid respectively. In order to establish whether or not this difference of Michaelis constants existed, it was considered desirable to use a different experimental approach, rather than to multiply the number of direct Michaelis constant determinations. A method involving mixed substrates was devised, the theoretical basis of which has previously been given (Thorn, 1949*b*). A simpler method, based on the same extension of the Michaelis-Menten theory, has also been described by Whittaker & Adams (1949).

The action of the succinic oxidase system on mixtures of succinic acid and tetradeuterosuccinic acid. The rate of oxygen consumption of the system was measured in the presence of a constant total concentration of substrate. In each experiment one flask contained succinic acid alone as substrate, one contained tetradeuterosuccinic acid alone, and the remainder contained mixtures of the two acids in various proportions. Fig. 5 shows the experimental points representing the average of four experiments. The curves in Fig. 5 were calculated by means of the mixed-substrate equation (Thorn, 1949*b*), which was simplified for this purpose as follows:

$$v_t=\frac{VxK'_m+V'x'K_m}{K_mx'+K'_mx+K_mK'_m},$$

where v_t is the total rate of disappearance of the two substrates when both are present in initial concentrations x, x'; V, V' are the 'maximum velocities' for the two substrates tested separately, and K_m, K'_m are the two Michaelis constants. It will be noticed from the figure that the experimental points do not lie on a straight line, and therefore the fact that the enzyme has different Michaelis constants with respect to the two substrates is established. The value of 1·83 for the ratio K_m^D/K_m^H, arrived at from the determined Michaelis constants of the two substrates, is seen to be too great: a value of 1·45 for the ratio fits the experimental points quite well. This implies that the 'affinity' of the enzyme

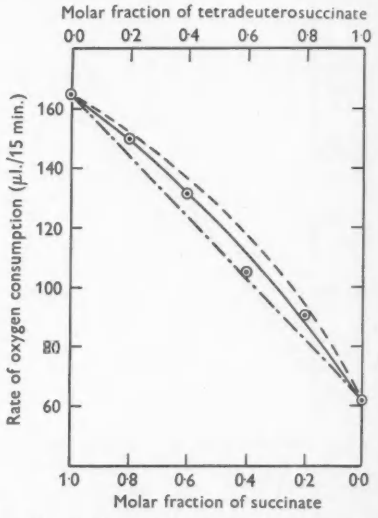

Fig. 5. The effect of the relative proportions of succinate and tetradeuterosuccinate on the rate of oxygen consumption of the succinic oxidase system. The points represent the average of four experiments. The lines are calculated from the mixed-substrate equation; - - - - -, $K_m^D/K_m^H=1{\cdot}0$; ——, $K_m^D/K_m^H=1{\cdot}45$; — — —, $K_m^D/K_m^H=1{\cdot}83$.

for succinic acid is 1·45 times its 'affinity' for tetradeuterosuccinic acid. It is considered that this ratio of affinities is more nearly correct than that which is calculated directly from the Michaelis constant determinations.

DISCUSSION

The results reported here may be summarized briefly by the statement that, using the particular enzyme preparation and the methods described, the succinic oxidase system oxidizes normal succinate faster than deuterium-substituted succinate, and appears to have a greater affinity for normal succinate than for deuterium-substituted succinate. The fact that the oxidation of tetradeuterosuccinate by this system is a slower reaction than the oxidation of normal succinate is in itself an interesting result, which might, however, have been anticipated from the results of previous work on base-catalysed proton

and deuteron transfer (Wynne-Jones, 1934; Wilson, 1936; Bonhoeffer, 1938) as well as on theoretical grounds (Eyring & Sherman, 1933). The observed difference between the activation energies of the reactions of tetradeuterosuccinate and normal succinate was found to be of the order of 1000 cal. Wilson (1936) has pointed out that this figure is to be expected on theoretical grounds from a consideration of the zero-point energies of carbon-hydrogen and carbon-deuterium bonds. Further discussion of the results from the point of view of the mechanism of the reaction becomes unprofitable unless it is certain that the rate-limiting step which was being measured was in fact the enzymic dehydrogenation of succinate. It is not, however, certain that this was the case.

It has been repeatedly pointed out (e.g. Keilin & Hartree, 1940, 1949; Slater, 1949*a*) that the activity of the succinic oxidase system depends to a large extent on the maintenance of the correct spatial arrangement of the various carriers concerned in the transfer of hydrogen or electrons from succinate to oxygen. The success of a method of preparation of the system can therefore be gauged by the extent to which the organization of the system in the cell is retained. It did not become evident until after this work had been carried out (Slater, 1949*b*) that the more recent preparation of the succinic oxidase system, introduced by Keilin & Hartree (1947), has a much higher activity than the former one (Keilin & Hartree, 1938), which was used in the studies described here. It can therefore be inferred that the older method of preparation interferes with the organization of the system to a greater extent than does the newer method. The importance of these considerations in the present investigation is that interference with the organization of the system may have caused some intermediate reaction to become the rate-limiting step, and the observed differences in reaction rate and activation energy for the normal and deuterated substrates may not represent the actual differences which would have been observed if the removal of hydrogen or deuterium from succinate had been the rate-limiting step. It is interesting to note in this connexion that the activation energy for the oxidation of succinate by the 1947 succinic oxidase preparation has recently been found by Slater (1950) to be 7520 cal., a figure markedly different from that obtained by Hadidian & Hoagland (1939) and in this investigation. It seems possible that the older Keilin & Hartree preparation and the preparation used by Hadidian & Hoagland (that of Stotz & Hastings, 1937) are comparable from the point of view of degree of interference with the organization of the system, and that they therefore give similar values for activation energy, whereas it is certain that the newer Keilin & Hartree preparation represents a more successful attempt to preserve the spatial arrangement of the carrier system transferring hydrogen or electrons from succinate to oxygen (Slater, private communication).

It is therefore necessary to call attention, not to the actual values which were found for the activation energies of the reactions of succinic and tetradeuterosuccinic acid, but to the difference which was found between these activation energies. Similarly, the values which were found for the Michaelis constants cannot be taken to represent the dissociation constants of enzyme-substrate complexes, even within the approximation imposed by the Michaelis-Menten theory, since the kinetics of the reaction appear to be too complicated to allow of such a simple interpretation. Attention should rather be drawn to the difference which appears to exist between the behaviour of succinate and tetradeuterosuccinate when this experimental method is used, and to the fact that such a difference is confirmed when the two substrates are allowed to compete.

A few general conclusions may be drawn about the use of deuterium in biochemical studies. Clearly, experiments in which deuterium is used as a tracer are justified from a qualitative point of view. Quantitative deductions, however, may be in error unless precautions have been taken, since the amount of deuterium or of a deuterated compound which has undergone reaction does not necessarily represent the amount of hydrogen or of a normal compound which would have reacted under the same conditions. The nature of the errors which might be incurred may be outlined.

The majority of tracer experiments with deuterium fall into two classes. In the first class deuterium is used as a label for a carbon chain. That is to say, a compound containing stably-bound deuterium is synthesized and is administered to an animal. It is of interest subsequently to isolate a series of compounds from the animal, and to determine in which compounds deuterium is present at a concentration higher than it is found to be in the body water of the animal. Such compounds can be said to have been derived more or less directly from the compound administered. The method is exemplified by a study of palmitic acid metabolism by Stetten & Schoenheimer (1940). The second class of experiment consists of enriching the body fluids of an animal with a small percentage of D_2O, usually to not more than about 3 %, so that all metabolic reactions take place in a medium of labelled hydrogen. The exchangeable hydrogen atoms of metabolic intermediates exchange with the labelled hydrogen of the medium, and the intermediates themselves become labelled with deuterium. In the course of subsequent reactions some of the deuterium introduced in this manner may become no longer exchangeable, and

the compounds so synthesized contain stably-bound deuterium. Deuterium may also be introduced by reduction. Substances isolated from an animal after this type of experiment, which contain stably-bound deuterium, must have been synthesized during the experiment. The work of Stetten and his co-workers on glycogen synthesis under various conditions (e.g. Stetten & Boxer, 1944) may be cited as examples of this type of experiment.

In the first class of experiment, deuterium transfer is not of interest, except in so far as an excess of D_2O appearing in the body water of the animal indicates that a proportion of the administered compound may have been largely metabolized. If, however, the enzymes concerned with the conversion of the compound administered have different affinities for normal and deuterated substrates, the amount of deuterated compound converted will not reflect the amount of normal compound converted under the same conditions. In the case of the mixed-substrate experiments reported here, it can be shown by means of the mixed-substrate equation, and using the ratio $K_m^D/K_m^H = 1{\cdot}45$, that in mixtures of succinate with up to about 20 % tetradeuterosuccinate, the amount of tetradeuterosuccinate undergoing reaction is about 80 % of what it would be if K_m^D was actually equal to K_m^H. It is of interest that Simmonds, Cohn & du Vigneaud (1947) found that the percentage of deuterium in the methyl groups of methionine administered to rats had no influence on the percentage of choline and creatine methyl groups derived from the methionine. It would seem that the enzymes responsible for these transmethylation reactions handle normal and deuterated substrates in the same way.

The case of experiments of the second class is more complex since, in addition to the possibility of different affinities of enzymes for normal and deuterated substrates, there arises the question of hydrogen and deuterium transfer reactions. In practice, however, it seems likely that the former possibility would be an unimportant source of error. In terms of the experiments reported here, an enrichment of 3 % D_2O in the body water of the animal would mean that the succinic system would be likely to encounter only occasional molecules of a deuterated succinic acid, and even then the acid would probably only contain one atom of deuterium. The ratio of the affinities of the system for monodeuterosuccinic acid and normal succinic acid must be very near to unity, and preferential oxidation of the normal substrate would be unlikely to occur. It remains to consider the question of proton and deuteron transfer reactions. There can be no doubt that deuteron transfer reactions are slower than the corresponding proton transfer reactions. Reviewing the question, Bonhoeffer (1938) found that in cases of base-catalysis (in which the rate-determining stage is the ionization of protons or deuterons from carbon atoms) proton transfer was in all cases from three to ten times faster than deuteron transfer. It is of further interest that Halford, Anderson, Bates & Swisher (1935) studied the base-catalysed reaction,

$$CH_3.CO.CH_3 + HOD \rightleftharpoons CH_2D.CO.CH_3 + HOH,$$

and found that the equilibrium constant was 2·1. In view of the different rates of proton and deuteron transfer, and this information about the equilibrium distribution of deuterium between water and acetone, it seems that the chance exists for unequal distribution of deuterium between body water and metabolites to occur in animals. The point may be raised, and indeed has been raised in this context (Schoenheimer & Rittenberg, 1936), that if unequal distribution of deuterium takes place between body water and metabolites, then enrichment of a hydrogen isotope should take place under normal circumstances. There is, however, no satisfactory evidence that such enrichment does take place in biological systems (see introductory discussion).

These paradoxical lines of evidence must presumably be harmonized by a consideration of the deuterium/hydrogen ratio in the experiments from which the evidence is drawn. Under natural conditions, the proportion of deuterium is so small that unequal distribution of deuterium between body water and metabolites would seem to be negligible, and within the limits of error of the methods of determination. If the proportion of deuterium is increased, there should be reached a stage when unequal distribution of deuterium between body water and metabolites exceeds experimental error, and above this critical level it would be no longer possible to draw quantitative conclusions unless a correction factor could be applied.

The experiments reported here were not designed to study equilibria of this nature, and discussion of this aspect of the problem cannot usefully be prolonged. Schoenheimer (1946) has stated that 'The living organism does not discriminate between isotopes of the same element, stable or radioactive, and treats all alike'. Sufficient evidence, however, seems to be available to make it necessary in the case of hydrogen and deuterium to qualify such a viewpoint with some reference to the proportion of the heavy isotope below which the statement is true within experimental error. Furthermore, as was pointed out in the introductory discussion, the fact that the metabolic rate of an animal is not influenced by a given concentration of deuterium in the body water does not seem to justify the assumption that unequal distribution of the isotope between body water and metabolites does not take place to a significant extent at that concentration of deuterium.

SUMMARY

1. Succinic acids were prepared, containing 77 atom % and 42 atom % of deuterium in the methylene groups.

2. Succinic acid containing 77 atom % D in the methylene groups was oxidized by a succinic oxidase preparation at about 40 % of the rate of oxidation of normal succinic acid under the same conditions. Succinic acid containing 42 atom % D in the methylene groups was oxidized at about 70 % of the rate of oxidation of normal succinic acid.

3. The activation energy of the reaction involving succinic acid containing 77 atom % D in the methylene groups was found to be 1450 ± 450 cal. higher than the activation energy of the reaction involving normal succinic acid.

4. The affinity of the succinic oxidase preparation for normal succinic acid was found to be about 1·45 times its affinity for succinic acid containing 77 atom % D in the methylene groups.

5. The results are discussed, particularly in relation to the use of deuterium as a tracer in biological experiments.

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REFERENCES

Anfinsen, C. (1946). *The Preparation and Measurement of Isotopic Tracers*, p. 61, edited by Wilson, D. W., Nier, A. O. & Rieman, S. P. Michigan: Edwards.

Barbour, H. G. (1934). *Proc. Soc. exp. Biol., N.Y.*, **32**, 1365.

Bonhoeffer, K. F. (1938). *Trans. Faraday Soc.* **34**, 252.

Dole, M. (1936). *J. Amer. chem. Soc.* **58**, 580.

Dungern, M. von (1936). *Z. Biol.* **97**, 187.

Emeleus, H. J., James, F. W., King, A., Pearson, T. G., Purcell, R. H. & Briscoe, H. V. A. (1934). *J. chem. Soc.* pp. 1207, 1948.

Erlenmeyer, H., Schonauer, W. & Süllmann, H. (1936). *Helv. chim. Acta*, **19**, 1376.

Eyring, H. & Sherman, A. (1933). *J. chem. Phys.* **1**, 345.

Goepfert, G. J. (1940). *Biochem. J.* **34**, 1012.

Hadidian, Z. & Hoagland, H. (1939). *J. gen. Physiol.* **23**, 86.

Haldane, J. B. S. (1930). *Enzymes*, p. 37. London: Longmans, Green and Co.

Halford, J. O. & Anderson, L. C. (1936). *J. Amer. chem. Soc.* **58**, 736.

Halford, J. O., Anderson, L. C., Bates, J. R. & Swisher, R. D. (1935). *J. Amer. chem. Soc.* **57**, 1663.

Hughes, A. H., Yudkin, J., Kemp, I. & Rideal, E. K. (1934). *J. chem. Soc.* p. 1105.

Keilin, D. & Hartree, E. F. (1937). *Proc. roy. Soc.* B, **122**, 298.

Keilin, D. & Hartree, E. F. (1938). *Proc. roy. Soc.* B, **125**, 171.

Keilin, D. & Hartree, E. F. (1940). *Proc. roy. Soc.* B, **129**, 277.

Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.

Keilin, D. & Hartree, E. F. (1949). *Biochem. J.* **44**, 205.

Keston, A. S., Rittenberg, D. & Schoenheimer, R. (1937). *J. biol. Chem.* **122**, 227.

Lewis, G. N. (1933). *J. Amer. chem. Soc.* **55**, 3503.

Linderstrøm-Lang, K., Jacobsen, O. & Johansen, G. (1938). *C.R. Lab. Carlsberg*, **23**, 17.

Linderstrøm-Lang, K. & Lanz, H., jun. (1938). *C.R. Lab. Carlsberg*, **21**, 315.

Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.

Moureu, C. & Bongrand, J. C. (1920). *Ann. Chim.* (Sér. 9), **14**, 5.

Pacsu, E. (1934). *J. Amer. chem. Soc.* **56**, 245.

Schoenheimer, R. (1946). *The Dynamic State of Body Constituents*, 2nd ed., p. 6. Cambridge, Mass.: Harvard University Press.

Schoenheimer, R. & Rittenberg, D. (1936). *J. biol. Chem.* **114**, 381.

Simmonds, J., Cohn, M. & du Vigneaud, V. (1947). *J. biol. Chem.* **170**, 631.

Slater, E. C. (1949*a*). *Biochem. J.* **45**, 8.

Slater, E. C. (1949*b*). *Biochem. J.* **44**, 305.

Slater, E. C. (1950). *Biochem. J.* **46**, 484.

Stetten, D., jun. & Boxer, G. E. (1944). *J. biol. Chem.* **155**, 231.

Stetten, D., jun. & Schoenheimer, R. (1940). *J. biol. Chem.* **133**, 329.

Stotz, E. & Hastings, A. B. (1937). *J. biol. Chem.* **118**, 479.

Taylor, G. W. & Harvey, B. N. (1934). *Proc. Soc. exp. Biol., N.Y.*, **31**, 954.

Thorn, M. B. (1949*a*). *Biochem. J.* **44**, xx.

Thorn, M. B. (1949*b*). *Nature, Lond.*, **164**, 27.

Weinmann, E. O., Morehouse, M. G. & Winzler, R. J. (1947). *J. biol. Chem.* **168**, 717.

Whittaker, V. P. & Adams, D. H. (1949). *Nature, Lond.*, **164**, 315.

Widmark, E. M. F. (1922). *Skand. Arch. Physiol.* **41**, 200.

Wilson, C. L. (1936). *J. chem. Soc.* p. 1550.

Wynne-Jones, W. F. K. (1934). *J. chem. Phys.* **2**, 381.

Wynne-Jones, W. F. K. (1935). *Chem. Rev.* **17**, 115.

Synthesis of Milk Fat from Acetic Acid ($CH_3{}^{14}COOH$) by the Perfused Isolated Bovine Udder

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The technique of perfusion of the isolated udder is likely to give valuable information on the mechanisms of milk formation, provided it can be shown that most of the milk collected is actually a product of the secretory activity of the gland during the experiment. This point is not easy to demonstrate by ordinary chemical methods, because present-day techniques only permit of a rather limited perfusion period, and also because in the relatively large quantity of tissue involved the various precursors, intermediates and products are distributed in an unknown manner. Moreover, although some of the milk held in the alveoli and finer branches of the duct system can be collected via the teat after administration of oxytocin, the difficulty remains that the proportion of preformed to newly formed constituents in any milk sample must remain uncertain.

Two modern methods for perfusing the isolated bovine udder have so far been described (Petersen, Shaw & Visscher, 1939; Peeters & Massart, 1947), and a number of studies, mainly on the physiology of milk ejection, have been carried out by means of these techniques. The demonstration that the udder of the intact goat synthesizes milk fatty acids from ^{14}C-labelled acetate (Popják, French & Folley, 1950, 1951) suggested the possibility of a direct test of the ability of the isolated preparation to synthesize milk constituents in the normal manner.

The results presented in this paper show that the perfused, isolated udder can use $CH_3{}^{14}COONa$ added to the perfusion fluid for synthesis of milk constituents, in particular fatty acids. They show further, however, that the conditions of perfusion need to be most carefully controlled if comparisons are to be made with processes occurring in the living animal. For this reason the interpretation of the results, in terms of the synthetic mechanisms occurring in the isolated organ, must be regarded as tentative at present.

EXPERIMENTAL

The udder of a lactating cow (East Flemish breed), yielding about 12 kg. milk daily, was perfused for 2 hr. by the method of Peeters & Massart (1947). An important feature of this technique is that it allows the simultaneous perfusion of two separated udder halves, each consisting of two mammary glands, with no mixing of the blood in the two circulations, one udder half can thus serve as control for the other. In this experiment the left udder half was perfused with blood containing $CH_3{}^{14}COONa$ while $NaH^{14}CO_3$ and heavy water (D_2O) were added to the blood perfusing the right half. Since it was expected that radioactive CO_2 would be given off continuously to the atmosphere in the artificial lungs, the apparatus was set up in the open air, and the experimenters wore service respirators fitted with soda-lime canisters.

Care was taken to choose a cow with a normal udder, showing on clinical examination no evidence of mastitis. Immediately before slaughter the cow was milked out as completely as possible with the aid of intravenously injected oxytocin. The udder, removed as soon as the cow was shot, weighed approximately 20 kg. It was perfused with saline to remove blood, completely bisected along the median septum and the halves connected to the perfusion apparatus. The teats were cut off at the tip and cannulae inserted, residual milk being allowed to drain from the cisterns; this milk was discarded.

Approximately 3 l. heparinized fresh cow blood was initially used for the perfusion of each udder half, but owing to the unusually large size of the udder it was necessary to add a further 2 l. blood to each half in the course of the first 30 min. The following additions to the perfusion bloods were made (at the outset unless otherwise stated): left ('acetate') side: 1·8 g. glucose, 600 mg. sodium acetate containing 1 mc. $CH_3{}^{14}COONa$, 80 ml. physiological saline, 25 mg. $NaHCO_3$ in 200 ml. physiological saline added by means of a constant-drip device to the arterial (i.e. oxygenated) blood continuously throughout the perfusion; right ('bicarbonate') side: 1·8 g. glucose, 600 mg. sodium acetate, 80 ml. D_2O (99·5%) containing NaCl to 0·9%, 1 mc. $NaH^{14}CO_3$ (25 mg.) in 200 ml. physiological saline added continuously to the arterial blood as for the left side. It is thus seen that the chemical composition of the bloods used for the perfusion of both udder halves was quantitatively identical. The addition of the bicarbonate

was spread over the whole perfusion period because if it were all added at the outset most of the labelled CO_2 would soon be lost in the artificial lung. The purpose of the addition of glucose and carrier acetate to the blood was to ensure a supply of milk precursors and energy-yielding substrates.

Samples of venous blood from each circulation were taken at the beginning of the perfusion, at 15 min. and at subsequent 30 min. intervals, for determination of CO_2 content and specific activity, and for determination of the D content of the blood water. These samples were at once sealed into ampoules and kept frozen solid until analysis. A measured volume, 0·25–0·50 ml., of thawed blood was quickly pipetted into a bulb connected with a high-vacuum train, the CO_2 liberated with lactic acid and its volume measured, after suitable purification, in a McLeod gauge. It was then introduced into an internal gas counter for radioactivity measurement. No special precautions were taken to prevent loss of gas during sampling or pipetting so that the blood CO_2 values can only be regarded as approximate.

The milk secreted by each half during the perfusion was collected, the udder halves being emptied by oxytocin at the end of the perfusion; adequate samples were frozen solid and kept in the frozen state until preparation of fatty acid fractions could be begun. Samples of udder tissue were taken from each half at the end of the perfusion, frozen and kept for preparation and fractionation of tissue fat.

Unfortunately, for a reason unknown, oxygenation and CO_2 clearance was poor on the left ('acetate') side. This was reflected in high blood CO_2 concentrations as compared with blood from the right side (see Table 1) and was probably responsible for the fact that only 140 ml. milk were secreted during the 2 hr. perfusion by the left udder half as compared with 1420 ml. secreted by the right half. The fat content of milk samples from both udder halves was practically the same, 4·7 and 4·75%.

Crude glyceride fatty acid fractions (water-soluble and insoluble, steam-volatile; non-volatile solid; non-volatile liquid acids) and some chromatographically pure fatty acids and also cholesterol were prepared from the samples of milk and udder tissue as described previously (Popják, French & Folley, 1951; Popják, French, Hunter & Martin, 1951). The distributions of the fractions obtained from each of the four samples are given in Table 2. The specific activities of the fatty acid fractions from the udder half perfused with $CH_3{}^{14}COONa$ were determined as before on solid preparations of salts using an end-window counter; the much less active fractions from the right side were combusted in O_2, the CO_2 purified and counted in a gas counter. The water resulting from the combustion was purified and its D content determined by the falling-drop method (see Popják, 1950).

RESULTS

Blood CO_2. The CO_2 content of blood samples taken at various times during the perfusion and the specific activities of the CO_2 are given in Table 1. During the first 15 min., the CO_2 content of blood from the left ('acetate') side was within normal limits for venous blood of the lactating ruminant (cf. figures for the goat given by Graham, Houchin, Peterson & Turner, 1938), but thereafter rose progressively to a high maximum value at 90 min. However, the CO_2 content of the sample taken at the end of the perfusion (120 min.) was back within the normal range. These results illustrate the fact, already referred to, that the artificial lung on this side of the apparatus was not working efficiently during the perfusion. It was also observed that the blood in this half of the apparatus was haemolysed to some extent. Whether this was a result or a cause of the poor oxygenation and consequent retention of CO_2 is not known.

Table 1. *Amount and specific activity of* CO_2 *in blood samples obtained during perfusion of lactating bovine udder*

Sample no.	Time (min.)	Vol. % CO_2 at N.T.P.	Specific activity of CO_2 (1×10^{-3} μc./mg. C)
	I. Left side: 1 mc. $CH_3{}^{14}COONa$		
1	0	44·4	147·1
5	15	39·4	39·0
7	30	109·0	30·9
9	60	111·6	31·4
11	90	210·0	21·0
14	120	54·5	43·7
	II. Right side: 1 mc. $NaH^{14}CO_3$		
3	0	48·0	0·0
6	15	87·2	3·5
8	30	94·4	9·1
10	60	63·4	11·7
12	90	55·6	29·0
16	120	42·2	121·5

The CO_2 content of the blood samples from the right ('bicarbonate') side showed some tendency to increase during the early stages of the perfusion, but the maximum value reached was not excessive and the values declined to normal in the later stages. Evidently oxygenation and CO_2 clearance were satisfactory on this side. The blood passing through this artificial lung underwent no haemolysis.

The highest specific activity of CO_2 obtained from blood taken from the left ('acetate') side was found in the sample taken at zero time. This sample was taken from venous blood immediately after the circulation through the udder half had been established, and the fact that the specific activity of its CO_2 was the greatest observed in the series illustrates the rapidity of the uptake and oxidation of acetate by the lactating mammary gland under the conditions of this experiment. In the intact animal the excretion is slower, the maximum specific activity of the respiratory CO_2 following the injection of $CH_3{}^{14}COONa$ occurring at 12–15 min. in the goat (Popják, French & Folley, 1950, 1951) and at 10 min. in the cow (Kleiber, Smith & Tolbert, 1950). As might be expected from the manner in which the labelled bicarbonate was added to the perfusion blood, the specific activity of blood CO_2 from the right side was low at the outset and rose slowly during the earlier stages, but more rapidly

later on, and attained its highest value at the end of the perfusion period.

Fatty acids. The distribution of glyceride fatty acids in the milk and udder tissue (Table 2) showed that the composition of the fats obtained from both halves of the perfusion was normal. The specific activities of the four crude fatty acid fractions prepared from milk fat secreted by the left udder half,

Table 2. *Distribution of glyceride fatty acids in samples of milk and udder fat obtained from perfusion experiment*

Fraction	Milk fat: Amount* isolated (g.)	Milk fat: % of total	Udder fat: Amount* isolated (g.)	Udder fat: % of total
I. Left side: 1 mc. $CH_3{}^{14}COONa$				
Volatile:				
Water-soluble	0·457	7·7	0·347	3·0
Water-insoluble	0·139	2·3	0·159	1·4
Non-volatile	5·36	89·9	11·12	95·6
II. Right side: 1 mc. $NaH^{14}CO_3$				
Volatile:				
Water-soluble	1·08	6·5	0·263	4·5
Water-insoluble	0·209	1·3	0·155	2·6
Non-volatile	15·3	92·1	5·46	92·8

* The amounts isolated were samples and do not represent total yields. The milk obtained from the left side (140 ml.) contained 4·70% and that from the right side (1420 ml.) 4·75% fat. The udder tissues from each half, weighing 10 kg. each, contained 5·0% glyceride fat.

Table 3. *Specific activities of glyceride fatty acids, cholesterol and lactose from milk and udder tissue after perfusion of left half of udder with* $CH_3{}^{14}COONa$ (1 *mc.*) *and of right half with* $NaH^{14}CO_3$ (1 *mc.*)

(Specific activities are expressed as 1×10^{-3} μc./mg. C.)

Substance	Left side: Milk	Left side: Udder	Right side: Udder
Steam-volatile fatty acids:			
Water-soluble	3·020	31·450	0·053
Water-insoluble	1·590	14·890	0·006
Non-volatile fatty acids:			
Solid	0·334	2·390	0·001
Liquid	0·239	1·510	0·000
Cholesterol	0·028	0·126	—
Lactose	0·178	—	0·003*

* Isolated from the milk.

and from udder fat from both sides of the udder, are given in Table 3. On the left side there was definite incorporation of labelled C into fatty acids belonging to all four fractions, the mean specific activities of the fractions falling progressively with increase in the mean chain length. The specific activities of similar fractions isolated from fat obtained from the tissue of this udder half were in all four cases some 7–10 times greater than those of the corresponding fractions from the milk. This may be ascribed to dilution of the milk fat with the fat of preformed milk existing in the alveoli at the beginning of the perfusion and which it was not possible completely to remove from the udder tissue even by intravenous injection of oxytocin followed by the most thorough milking-out. A rapid turnover of fatty acids in the epithelial cells themselves would also contribute to the observed difference between the specific activities of the fatty acid fractions from milk and udder tissue respectively.

Table 4. *Specific activities of pure fatty acids isolated from udder tissue perfused with blood containing* 1 *mc. of* $CH_3{}^{14}COONa$

Fatty acid	Specific activity (1×10^{-3} μc./mg. C)
Butyric	43·50
Caproic (hexanoic)	27·00
Caprylic (octanoic)	24·20
Capric (decanoic)	21·90
Lauric (dodecanoic)	19·03
Myristic (tetradecanoic)	12·48
Palmitic (hexadecanoic)	3·69
Stearic (octadecanoic)	0·045
Oleic (octadec-9-enoic)	0·030

The specific activities of purified fatty acids isolated from the tissue fat of the udder half perfused with $CH_3{}^{14}COONa$ are given in Table 4. It will be seen that the specific activities decrease as the chain length increases from butyric acid upwards.

Table 5. *Deuterium content of milk lactose and of fatty acid fractions isolated from udder tissue perfused with blood containing* D_2O

Fraction	D (atoms % excess)
Steam volatile fatty acids:	
Water-soluble	0·02
Water-insoluble	—
Non-volatile fatty acids:	
Solid	<0·01
Liquid	<0·01
Lactose	<0·02
Blood water	0·77
Milk water	0·75

By contrast with the above results, the specific activities of fatty-acid fractions obtained from fat from the right ('bicarbonate') udder half were in all cases very low (in fact only measurable by gas-counting technique), showing that incorporation of $^{14}CO_2$ into fatty acids in the mammary gland under these conditions is negligible. Thus, in the case of the water-soluble steam-volatile fatty acids, the specific activity of the udder fat from the right side was only about 1/1000 of that from the left side, the contrast being pointed by the fact that milk was secreted about ten times faster by the right udder half than by the left udder half.

The results of deuterium determinations on the fatty acid fractions isolated from the 'bicarbonate' udder half are given in Table 5. The incorporation of deuterium was in all cases very low.

Cholesterol. Cholesterol isolated both from milk and udder tissue from the left ('acetate') udder half was radioactive, the specific activity of the cholesterol from the udder being about four times that of the milk cholesterol. The results, in confirmation of previous findings on the rabbit and goat *in vivo* (Popják & Beeckmans, 1950; Popják, French & Folley, 1950, 1951), indicate synthesis of cholesterol in the isolated udder tissue.

DISCUSSION

In planning the experiment it was assumed that each perfused udder half would secrete milk at the rate of about 100 ml./hr. and that this milk would be formed from material taken up at a fairly steady rate from the blood, the chemical composition of the alveolar tissue of the udder itself remaining constant. Such a process would presumably approximate to normal secretion. The results of the experiment were, however, quite different. The right ('bicarbonate') half of the udder produced 1420 ml. of milk containing 4·75% fat, i.e. a total of 67·45 g. of fat. The amount of glucose and total fatty acids in the 5 l. of perfusion blood was certainly less than a third of this quantity. Most of this milk fat must therefore have arisen from other sources, presumably from the udder tissue itself. As regards the left ('acetate') side also there is evidence that the metabolites supplied were insufficient for the needs of the tissue. The specific activity of the blood CO_2 reached a peak immediately after the perfusion began, after which it dropped sharply and thereafter remained practically constant until the end of the experiment. It seems probable that the gland cells had taken up most of the acetate from the blood in a very short time, other metabolites probably being removed equally rapidly. It is calculated from the yields of udder and milk fatty acid fractions and their specific activities that a large fraction of the ^{14}C administered as acetate, nearly 400 μc., i.e. 40%, was present in the fatty acids at the end of the experiment. The most likely interpretation of the time course of the blood CO_2 specific activity would therefore seem to be that the initial high value was due to oxidation of free acetate, whereas the later steady excretion of $^{14}CO_2$ was due to oxidation of reserve materials in the tissue, including the labelled fatty acids formed from acetate. The fact that after the first 15 min. the specific activity of the blood CO_2 on the left ('acetate') side (Table 1) was of the same order as that of the volatile fatty acid fractions isolated from the udder (Table 3) is in keeping with this suggestion. It is also probable that the initial perfusion with saline had the effect of depleting the reserves of metabolites in the cells, so that the available material in the blood was rapidly taken up. Beyond this point the two halves behaved differently: on the right side, where oxygenation and CO_2 removal were adequate, a large amount of milk was secreted, whereas on the left side only small amounts of the labelled fatty acids formed in the udder found their way into the milk.

The general conclusion to be drawn from these considerations is that the tissue was active in the turnover and secretion of fat, but it is very doubtful whether net fat synthesis occurred at all on the left side, and it probably occurred only to a limited extent on the right side. On the whole it is probable that breakdown of tissue fat by oxidation was the predominant process. The R.Q. of the udder can be taken as a measure of net fat synthesis or breakdown (see Folley, 1949). It is of interest that although the R.Q. of the lactating bovine udder *in vivo* has been generally found to be greater than unity, Shaw (1939) reported a mean value of only 0·8 for the perfused, isolated udder. It is known that the R.Q. of tissue slices of lactating mammary gland is very sensitive to the supply of suitable substrates in the medium (Folley & French, 1949, 1950). Further work with the perfused preparation should aim at the maintenance of continuous net synthesis of milk constituents, which might be possible by maintenance of constancy of composition in the perfusion medium.

In spite of these differences from physiological conditions, some qualitative conclusions can be drawn from the results. The secretion of radioactive milk fatty acids by the udder perfused with $CH_3{}^{14}COOH$ in general confirms previous results on the intact lactating goat (Popják, French & Folley, 1950, 1951) which led to the conclusion that the lactating ruminant udder brings about the synthesis of milk fat by condensation of acetate or some C_2 unit derived therefrom. The specific activities of all four crude fractions of milk fatty acids in this experiment were considerably lower than the maximum values found in the experiment on the live goat. The fatty acids isolated from the udder tissue, however, had an activity comparable to that found in goat milk several hours after the administration of labelled acetate. It is probable that the fatty acids synthesized in the cells are the direct precursors of the milk fatty acids, and the high activity of this fat, which includes 'structural' fat of the cells, illustrates the previous conclusion (Popják, French & Folley, 1951) that milk-fat synthesis is the end result of the dynamic turnover of the udder cell constituents.

The decrease of specific activity of the milk and udder fatty acids with increasing chain length indicates, in agreement with the results obtained on the intact lactating goat, that the short-chain acids could not have arisen to any considerable extent by the direct degradation of long-chain acids, mainly

oleic, as required by the theory of Hilditch (see Achaya & Hilditch, 1950). There appears to be a significant difference between the order of the specific activities of the pure fatty acids found in this experiment compared with those in the intact animal. It has been reported (French, Hunter, Martin & Popják, 1951; Popják, French, Hunter & Martin, 1951) that in the lactating goat during the first 12 hr. after the injection of labelled acetate, the specific activities of the milk fatty acids increased with chain length from butyric up to capric (decanoic). These results, together with those obtained by chemical degradation of some of the acids, could be explained only by the assumption that butyric acid synthesized in the gland from the labelled acetate was continuously being diluted with a non-isotopic C_4 compound. It was suggested that this C_4 precursor was obtained from the blood and that it might be β-hydroxybutyric acid. In the absence of this unlabelled precursor it would be expected that in the early part of the experiment the butyric acid would be the most active product of the condensation of C_2 units. This expectation appears to apply in the present experiment, in which the synthetic period was short, and the supply of β-hydroxybutyric acid in the perfusion blood was probably very small.

The results of the present experiment reinforce the previous conclusions drawn from the *in vivo* work, in that they exclude possible complications due to the incorporation in the liver or other organs of ^{14}C into carbohydrate or fat which could be transported to the udder, and there used as substrates for milk formation. Moreover, since the incorporation of $^{14}CO_2$ into milk fat in the mammary tissue has been found to be negligible, another factor which might complicate the interpretation of the previous tracer results can be disregarded. Kleiber *et al.* (1950) also found a low incorporation of $^{14}CO_2$ into milk fat in the intact cow.

The low values observed for incorporation of D into milk and udder fatty acids is not surprising when the turnover of the large quantities of fat present in the udder is taken into account. A significant proportion of D in the milk fat could only be expected if this fat arose directly from small molecules without dilution by preformed udder tissue fat. In order to study the uptake of D from body water, higher D_2O concentrations or longer experiments would be necessary.

The observed specific activities of milk and udder cholesterol agree with the conception of the turnover of udder constituents. Those constituents which are present in high concentration in the milk compared with the udder (the volatile fatty acids) are synthesized with high specific activity immediately following the administration of a single dose of precursor, whereas cholesterol, which has a higher concentration in the udder than in the milk, has a much lower activity, indicating a prolonged turnover time. It remains to be shown whether the low activity of stearic and oleic acids, found in this experiment as well as in the intact goat, can be entirely explained by this mechanism.

The lactose isolated from the milk on the left ('acetate') side had a significant radioactivity. Since extremely little activity was found in the milk lactose from the right ('bicarbonate') side, it appears that some pathway other than CO_2 fixation is responsible for the incorporation of acetate carbon into carbohydrate in the bovine udder. On the other hand, Kleiber *et al.* (1950) found that, following the injection of $Na_2{}^{14}CO_3$ into a lactating cow, lactose had the highest specific activity of the major milk constituents. In view of our results with the perfused, isolated udder, it seems probable that this was due to incorporation of $^{14}CO_2$ into glycogen in the liver followed by the transport of ^{14}C-glucose to the udder and its utilization for lactose formation.

SUMMARY

1. Two separated halves of a lactating cow udder were respectively perfused with blood containing carboxyl-labelled acetate (1 mc. $CH_3{}^{14}COONa$; 'acetate' half) and labelled bicarbonate (1 mc. $NaH^{14}CO_3$; 'bicarbonate' half) by a technique which permits of separate perfusion with no mixing of blood in the two circulations. Heavy water (D_2O) was added to the blood containing $NaH^{14}CO_3$.

2. The specific activity of the blood carbon dioxide in the 'acetate' udder half was maximal at the outset and thereafter fell to a fairly constant level throughout the rest of the experiment. Because of this and the fact that about 40% of the added ^{14}C was found in the udder fat at the end of the experiment, it would seem that the initial high specific activity of the blood carbon dioxide was due to the oxidation of free acetate, which was probably taken up rapidly by the udder tissue, the subsequent lower values corresponding to the oxidation of udder constituents, mainly fatty acids, into which the $CH_3{}^{14}COONa$ had been incorporated.

3. ^{14}C was incorporated into fatty acid fractions isolated from the milk and udder glycerides of the 'acetate' half, the mean specific activity falling with increasing mean chain length. The conclusion from previous work on a living lactating goat, that the udder utilizes acetate as a substrate for the synthesis of milk fatty acids, is therefore confirmed. The specific activities of the udder fat fractions were higher than those of corresponding fractions from the milk. This could be explained by dilution of newly synthesized fat by fat already existing in the udder tissue and by a rapid turnover of fat in the epithelial cells. The specific activities of pure fatty acids

isolated from the udder fat fell progressively with increasing chain length up to stearic and oleic acids. The short-chain acids could therefore not have been formed directly by degradation of long-chain acids.

4. The mean specific activities of fatty acid fractions isolated from udder fat from the 'bicarbonate' half were very low, indicating negligible incorporation of $^{14}CO_2$. The deuterium content of these fractions was also low, probably due to dilution of newly formed fat.

5. Cholesterol isolated from milk and udder fat from the 'acetate' half was radioactive, the specific activity of the udder cholesterol being higher than that from the milk. This confirms previous work on the goat indicating that cholesterol is synthesized in the udder.

6. Lactose isolated from milk from the 'acetate' half was appreciably radioactive, while milk lactose from the 'bicarbonate' half was not. The udder tissue must therefore incorporate acetate carbon into lactose by some mechanism other than carbon dioxide fixation.

7. The application of the udder perfusion technique to biochemical studies of milk formation is discussed in the light of the results of this experiment, and it is concluded that the perfusion conditions must be carefully controlled if valid comparisons are to be made with the lactating udder *in vivo*.

REFERENCES

Achaya, K. T. & Hilditch, T. P. (1950). *Proc. roy. Soc.* B, **137**, 187.

Folley, S. J. (1949). *Biol. Rev.* **24**, 316.

Folley, S. J. & French, T. H. (1949). *Biochem. J.* **45**, 117.

Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.

French, T. H., Hunter, G. D., Martin, A. J. P. & Popják, G. (1951). *Biochem. J.* **48**, vi.

Graham, W. R., jun., Houchin, O. B., Peterson, V. E. & Turner, C. W. (1938). *Amer. J. Physiol.* **122**, 150.

Kleiber, M., Smith, A. H. & Tolbert, B. (1950). *XVIIIth Int. physiol. Congr.*, Abstr., p. 304.

Peeters, G. & Massart, L. (1947). *Arch. int. Pharmacodyn.* **74**, 83.

Petersen, W. E., Shaw, J. C. & Visscher, M. B. (1939). *J. Dairy Sci.* **24**, 139.

Popják, G. (1950). *Biochem. J.* **46**, 558.

Popják, G. & Beeckmans, M.-L. (1950). *Biochem. J.* **46**, 547.

Popják, G., French, T. H. & Folley, S. J. (1950). *Biochem. J.* **46**, xxviii.

Popják, G., French, T. H. & Folley, S. J. (1951). *Biochem. J.* **48**, 411.

Popják, G., French, T. H., Hunter, G. D. & Martin, A. J. P. (1951). *Biochem. J.* **48**, 612.

Shaw, J. C. (1939). *J. Dairy Sci.* **22**, 438.

The Turnover Number of Cholinesterase

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Many problems in cholinesterase kinetics could be greatly simplified by a knowledge of the concentration of enzyme in the system being studied. It would, for instance, be of value to know the concentration of enzyme in various tissues, the blood in particular.

The concentration of enzyme in any system can be calculated from the activity if the turnover number (i.e. the number of molecules of substrate transformed in unit time/mol. of enzyme) is known. The turnover number (T) cannot be determined unless the enzyme is obtainable in a high degree of purity. Cholinesterase is not easily purified; in fact no pure preparation has been isolated from mammalian sources.

Easson & Stedman (1936), in discussing the fact that certain drugs, such as physostigmine, inhibit cholinesterase in very minute amounts, assumed that 'one molecule of drug will inhibit the activity of one unit or active centre of enzyme', and based their calculation of active-centre concentration on this assumption, together with the demonstration that these drugs behaved non-competitively. They found that horse serum cholinesterase had a T of 1490 mol. of acetylcholine/sec./active centre.

The theory of inhibition by means of a bimolecular reaction between active centre and inhibitor was furthered by Straus & Goldstein (1943), but the validity of the turnover number found, 450 mol./sec., was later denied by Goldstein (1944), since physostigmine behaved competitively. Augustinsson & Nachmansohn (1949) confirmed Goldstein's

observation that the inhibitors in question are competitive, although with a comparatively low dissociation constant.

The discovery of certain irreversible inhibitors of the phosphate ester type has enabled a fresh attack to be made on the problem. These inhibitors appear to be highly specific for cholinesterase (Mackworth & Webb, 1948; Mazur, 1946; Mazur & Bodansky, 1946). There is strong evidence that they react bimolecularly with enzyme active centres (Boursnell & Webb, 1949; Jansen, Nutting & Balls, 1948, 1949). If the assumptions are made that these inhibitors are in effect specific reagents for cholinesterase, and that an irreversible bimolecular reaction occurs, they may be used to titrate the active centres by means of an appropriate technique.

THEORETICAL

Determination of T

In this paper the turnover number will be given simply as a number, the number of molecules of acetylcholine hydrolysed/min./active centre at 38° and pH between 7·2 and 7·4 in the presence of Krebs-Henseleit saline. The acetylcholine concentrations are, for acetylcholinesterases, 3×10^{-3} M; for butyrylcholinesterases, 6×10^{-2} M. The types of enzyme will be designated as AChE and BChE respectively.

The unit of enzyme activity is 1 μl. carbon dioxide/30 min. A preparation of activity a units therefore hydrolyses $1{\cdot}49a \times 10^{-9}$ mol. monobasic ester/min. Assuming that 1 mol. inhibitor reacts with 1 mol. active centre, then on completion of the reaction, loss of activity L is equivalent to the quantity of inhibitor added. Therefore, if I is the molar concentration of inhibitor and v the volume in ml. of the system, L is equivalent to $Iv \times 10^{-3}$ mol. of enzyme. This quantity of enzyme hydrolyses $L \times 1{\cdot}49 \times 10^{-9}$ mol. of ester/min. Therefore 1 mol. active centre hydrolyses

$$\frac{1{\cdot}49L \times 10^{-6}}{Iv} \text{ mol. of ester/min.} = T \text{ (turnover no.).} \quad (1)$$

Experimental results can be expressed as the ratio of L to I such that $b = L/I$. Substituting in equation (1) this becomes

$$T = \frac{1{\cdot}49b \times 10^{-6}}{v}.$$

The calculation of b and its error is particularly simple, since on hypothesis the slope passes through the origin, and the complication of calculating the regression about an origin whose coordinates are the mean values of L and I is not necessary (Snedecor, 1946).

Determination of the active centre concentration

Once T is known, the active centre concentration may be calculated from the activity.

Let m be the molar concentration of active centre. The system of volume v thus contains $mv \times 10^{-3}$ mol. As before, the activity is $1{\cdot}49a \times 10^{-9}$ mol. ester/min. 1 mol. active centre hydrolyses T mol. ester/min. Therefore system hydrolyses $mvT \times 10^{-3}$ mol. ester/min.

Therefore

$$mvT \times 10^{-3} = 1{\cdot}49a \times 10^{-9},$$

whence

$$m = \frac{1{\cdot}49a \times 10^{-6}}{vT}. \quad (2)$$

If the substrate concentration used is not that at which T was determined, the activity may be adjusted by means of a standard activity-substrate concentration curve.

This treatment involves the assumption, in addition to those made, that the inhibitor is not involved in side reactions such as spontaneous hydrolysis, or hydrolysis by an enzyme as described by Mazur (1946). The inhibitor di*cyclo*hexyl fluorophosphonate (DCFP) is extremely resistant to spontaneous hydrolysis (Chapman & Saunders, 1948). It has, therefore, been used to determine the turnover number of the cholinesterase in the erythrocytes and plasma of various species.

EXPERIMENTAL

Preparation of enzymes from blood

Blood was collected in tubes containing solid oxalate. Plasma was separated by centrifuging, and the cells were washed twice with isotonic saline. Plasma or cells were diluted to the extent required to give activities of about 100–150 μl. CO_2/30 min., usually with distilled water, but for some experiments with Krebs-Henseleit saline.

Purification was effected by one of two methods. In the first, due to Davies & Smellie (1949), the cells were lysed, the stroma precipitated at pH about 6, and washed with distilled water and phosphate buffer till the liquor was colourless. The precipitate was then dispersed at pH 8 in Krebs-Henseleit saline. The purification was about 12-fold, and the separation of aliesterase (measured with tributyrin) poor. In the second method, the stroma, purified in a similar manner, was further purified by treatment with kieselguhr. For details see Mounter & Whittaker (1950).

The final suspension was in Krebs-Henseleit saline. The purification was about 30-fold, and aliesterase was completely removed.

Horse serum was purified by the first stage of Strelitz's (1944) method.

Estimation of enzyme activity and effect of inhibitors

Enzyme activities were measured in the Warburg apparatus, and calculated from four readings (Aldridge, Berry & Davies, 1949). In the case of acetylcholinesterase the ACh concentration was 3×10^{-3} M. Since at this concentration gas output is not linear after 15 min. from the time of mixing enzyme and substrate, readings were taken 5, 8, 11 and 14 min. after mixing. In the case of butyrylcholinesterases the ACh concentration was 6×10^{-2} M. Linear out-

puts were seldom observed, but the departure from linearity was slight and was ignored. Readings were taken at 10 min. intervals.

Inhibition was measured as follows: 0·4 ml. of enzyme preparation and 0·1 ml. of either distilled water or inhibitor in distilled water were placed in one side arm of a double side arm Warburg flask. In the other side arm was 0·5 ml. acetylcholine chloride in distilled water, and in the main compartment 2 ml. of Krebs-Henseleit saline. The flasks were gassed with 5% CO_2 in N_2 at 38° and incubated for 1 hr., preliminary trial having shown that no further inhibition occurred after this time, provided the extent of inhibition was not greater than 30–50%. A plot of loss of activity against concentration of inhibitor was in most cases linear, until such a degree of inhibition was reached, after which the loss of activity was less than expected from the higher concentrations. This is explicable in that the time of 1 hr. was insufficient to allow the higher concentrations of inhibitor to react to completion. In calculating T only the linear portion of the curve was used. At the end of the hour, both side arms were tipped, and readings taken. The reasonable assumption has been made that DCFP behaves like DFP with which, as Aldridge (1950) has shown, there is no reversal of inhibition on dilution. This procedure is therefore a legitimate device for speeding up the rate of inhibition.

The concentration of inhibitor reported is one-sixth that actually used in the side arm, because the enzyme solution was diluted to that extent in order to measure its activity. It is obvious that inhibitor concentration must also be referred to the entire volume in which the activity was measured.

Deviations from this procedure were required in two instances. (1) In order to check the possible deterioration on storage of our sample of DCFP, it was compared with a fresh sample of diethyl *p*-nitrophenyl phosphate (E600). Using the above technique, E600 inhibited to a far lesser extent. A consideration of the kinetics of the reaction, using the rate constant given by Aldridge (1950), showed that impossibly long incubation times, at least 15 hr., would be required. The enzyme preparations were not stable at 38° for more than 3 or 4 hr. Accordingly the enzyme and inhibitor were both concentrated tenfold, incubated for 2–2·5 hr. and the mixture diluted 1:10 into separate Warburg flasks for activity determinations. It was thus found that the T of partly purified human erythrocytes was the same for both inhibitors, which was taken as evidence that the DCFP had not deteriorated.

Table 1. *The purity of stored* DCFP *examined by comparison with fresh E* 600

(Turnover number of partially purified human erythrocyte ChE (Davies & Smellie).)

With DCFP	$1{\cdot}61 \pm 0{\cdot}09 \times 10^{-5}$	3 points on graph
With E600	$1{\cdot}63 \pm 0{\cdot}18 \times 10^{5}$	5 points on graph

(2) Some red-cell enzymes, notably those from dog and rat, required more than 1 hr. incubation to show linearity between loss of activity and inhibitor concentration; indeed dog cells failed to show linearity even after 5 hr. This shows that the red-cell enzyme of these species is inhibited more slowly than most.

RESULTS

The T of a partly purified human red-cell AChE preparation was, as shown in Table 1, 162,000. The T of partly purified horse serum BChE was 93,000, in excellent agreement with Easson & Stedman's value of 89,400.

That there should be a difference in the T of these two enzymes is not surprising in view of their different properties, especially in respect of substrate specificity. There is one other value with which these may be compared, that of Nachmansohn's electric eel AChE (Rothenberg & Nachmansohn, 1947). The T/mol. is 20,000,000; and if, as has been suggested (Nachmansohn, Rothenberg & Feld, 1948; Augustinsson & Nachmansohn, 1949) there are some 30 active centres/mol., the T/active centre is 670,000, a vastly different figure from human red-cell AChE. This observation suggested that there might be species differences between the AChE's and possibly also the BChE's. On the hypothesis that DCFP is a specific reagent, partial purification of tissue extracts should be unnecessary, provided that the ChE present is mainly if not wholly of one type. Nevertheless, a comparison was made between washed human red cells and a partly purified preparation from them of activity 700 μl. carbon dioxide/mg. protein/hr. (30-fold purification). Table 2 shows that the difference between them was small, thus indicating that a good approximation to the turnover number could be obtained without the necessity of carrying out an extensive programme of purification.

Table 2. *Effect of partial purification on turnover number of human erythrocyte ChE*

Enzyme	$T \times 10^{-5}$	No. of points	
Crude	$1{\cdot}94 \pm 0{\cdot}17$	4	$P = 0{\cdot}3$
Purified	$1{\cdot}67 \pm 0{\cdot}13$	6	

As a result of this observation, a survey was made of the turnover numbers of the red-cell enzyme of a number of species. Figures for human and dog plasma are included because these tissues, while showing moderate to high BChE activity, have a comparatively low AChE activity, as revealed by specific substrate tests (Mendel, Mundell & Rudney, 1943).

Table 3. *Turnover number of the blood ChE of different species*

Species	Plasma, BChE	Cells, AChE
Human	48,000	171,000
Horse	93,500*	13,000
Dog	99,000	50,000†
Rat	—	10,000

* Partly purified. † Lower limit.

This table shows that there are species differences among cholinesterases of the same type. This could be due to the fact that the ChE in each species is different, or it might be that the basic activity of the enzyme, which is the same in all species, is modified by its environment.

Preliminary studies on goat blood, which it was desired to study intensively, gave rather confusing results, in that each individual goat seemed to have its own T for red-cell AChE. It was not possible to keep a goat exclusively for bleeding, as Aldridge (1950) had done in earlier work, so that blood had to be drawn from such animals as were available. Accordingly, blood was drawn from ten goats, mostly females, and the following determinations carried out on the washed cells: normal ChE activity, normal aliesterase activity (as measured with tributyrin), ChE and aliesterase activities after 1 hr. inhibition by a given concentration of DCFP. This gave maximal inhibition of ChE. All samples were subject to precisely the same washing procedure, so that the results accurately reflect individual variation. The packed cells were diluted 1:4 with water. The results are given in Table 4.

There are no correlations or trends discernible in this table. On the assumption that DCFP is a specific reagent for ChE, the same loss of activity is to be expected regardless of the normal level. The variations in T should not exceed those expected from the regression method examined earlier. Tables 1 and 2 show that the coefficient of variation of T is between 5·4 and 8·8 %. (The exception, determined with E 600, involved the additional step of dilution, and may be disregarded.) Two separate 'regression' determinations of the same sample of goat cells gave coefficients of variation of 4·5 and 6·1 %. By contrast, the coefficient of variation of the T of Table 4 is 51·3 %. These variations are so much greater than the error of the method that it may be concluded that individual variations in susceptibility to inhibition exist. These variations are not entirely due to the fairly high sensitivity of aliesterase to inhibition, since there is no correlation between ChE loss and either normal aliesterase or loss of aliesterase. There is possibly some other component of comparable affinity for DCFP.

A similar examination was made of ten human bloods from fit young Service men. The results are given in Table 5. The mean T is $1{\cdot}87 \times 10^5$ and the coefficient of variation 22·3 %. Compared with the worst of the data of Tables 1 and 2, the variance ratio 6·4 with $n_1 = 9$ and $n_2 = \infty$, indicates individual variation greater than would be expected from the method of estimation.

Table 4. *Individual variations in turnover number of goat erythrocyte ChE*

			ChE activity (μl. CO_2/30 min.)			AliE activity (μl. CO_2/30 min.)	
Goat no.	Sex	DCFP $\times 10^{10}$	Normal	Loss	$T \times 10^{-4}$	Normal	Loss
114	F	5·97	76	23	1·91	71	2·5
410	F	5·97	73	38	3·16	67	3
123	M	5·97	67	23·5	1·95	66	7
—	—	5·23	103·5	48	4·56	67·3	5·5
161	F	6·6	70·5	47·5	3·58	80·5	5
J 102	F	6·6	116·5	12	0·90	106	15
J 110	F	5·47	78·5	13·5	1·23	88	15
J 117	F	5·47	127·5	28·5	2·59	84·5	2
J 125	F	5·47	71	15·5	1·41	91	6·5
J 171	F	5·79	120·5	54·5	4·68	90	6
Mean			**90·4** ±7·5		**2·62** ±0·44	**81·2** ±4·2	

Table 5. *Individual variations in turnover number of human erythrocyte ChE*

ChE (μl./30 min.)				Aliesterase (μl./30 min.)	
Normal	Loss	DCFP $\times 10^{10}$	$T \times 10^{-5}$	Normal	Loss
176	31·5	1·03	1·52	20	0
180	43·5	1·03	2·10	19	1
166	39	1·03	1·88	23	3
152	45	1·12	2·00	15	1
154	29·5	1·12	1·31	18·5	0
167	42	1·12	1·86	17	1
158	46·5	1·12	2·06	18	0·5
146	36	1·12	2·04	16	0
153	30	1·12	1·33	22	2·5
191	57·5	1·12	2·55	—	—

Table 6. *Effect of partial purification on turnover number of goat erythrocyte ChE*

Enzyme	Normal activity (μl. CO_2/30 min.)	Loss of activity	DCFP $\times 10^{-10}$ M	$T \times 10^{-4}$
Crude	129	40	5·34	3·7
Purified	83	68	5·34	6·3

In the light of this evidence we must re-examine the earlier statement, based on the comparison between crude and purified human cells, that the crude sample gives a good approximation to the T. The crude sample was taken from one person, whereas the purified sample was prepared from a bulk sample from six or eight persons. This comparison is now seen to be inadmissible. By contrast, the effect of purification on goat cells was studied on one sample, which was divided, part being taken for determination of T at one concentration of DCFP, the other part purified. The degree of purification was not measured except to note that the aliesterase activity was eliminated. The result is in Table 6. Purification has resulted in a marked increase in sensitivity to inhibition. It has thus been demonstrated that DCFP is by no means as selective as had originally been supposed.

DISCUSSION

The idea that an inhibitor might be practically specific for cholinesterase was first suggested by Easson & Stedman (1936). Although such inhibitors as physostigmine also inhibit liver esterase, they inhibit cholinesterase in such high dilution that at such dilution the effect on other esterases would be negligible. However, the possibility of reaction with inert proteins was indicated by the finding of Roepke (1937) that three to four times more physostigmine was required to produce a given inhibition in crude serum than in purified serum.

The specificity of di*iso*propyl fluorophosphonate appeared to be of a higher order. Mackworth & Webb (1948) found that it was thirty times as active as physostigmine against ChE, and concluded that 'fluorophosphonate has a very special affinity for the active centres attacked'. Mazur & Bodansky (1946) found that, within the limits of experimental error, the concentration giving 50% inhibition was the same for purified as for crude serum. Although Webb (1948) found that DFP was not specific for ChE, his data show that the next most sensitive enzyme studied, ox-liver esterase, required about 300 times as much DFP to give 50% inhibition. It was a reasonable supposition that on adding DFP or the chemically similar DCFP to a tissue extract the greater part of it would react with ChE, so that it would be in effect a specific reagent.

It appears, however, from the present results that tissue extracts may contain substances, such as aliesterase, which, while not greatly affected by DCFP have for it a sufficiently high affinity to interfere with its inhibition of ChE. The fact that goat red-cell aliesterase may interfere in this manner is consistent with the supposition that it is of low activity towards its substrate, but fairly reactive with DCFP.

A rather unexpected finding was the considerable individual variation in the susceptibility to inhibition of the ChE in tissue extracts. This raises the question of whether there may be individual variations in the net rate of inhibition. Both of these factors would be reflected in the commonly determined I_{50} values for irreversible inhibitors. Future kinetic studies must be designed to take account of this possibility, either by finding whether individual variations exist, or by using pooled material. Some of the data in Table 3 are unreliable, notably those for horse and dog cells, where the samples are from one animal.

The species variations noted in Table 3 are more likely to be due to the presence of substances competing with ChE for inhibitor than to substances modifying the basic activity of ChE. It should not be assumed that interfering substances have been removed by existing methods of purification, especially of red-cell enzyme, where the utmost degree of purification is only some 30-fold.

It is clear that DCFP cannot be used in the manner originally intended, to determine the T. The meaning of the numbers labelled T is obscure. It is not feasible to use them to calculate the concentration of ChE active centres, nor to attempt to estimate the capacity of the cells to combine with DCFP because we have no information about the combination of the inhibitor with other substances in the blood.

SUMMARY

1. A mathematical expression has been developed for calculating the turnover number of an enzyme, assuming the use of a highly specific irreversible inhibitor to determine the active centre concentration.

2. The following basic assumptions are involved: (*a*) the inhibitor is highly specific for the enzyme; (*b*) inhibition is irreversible; (*c*) the inhibition reaction is bimolecular.

3. This expression has been applied to the study of cholinesterases in tissue extracts, using the inhibitors di*cyclo*hexyl fluorophosphonate and diethyl *p*-nitrophenyl phosphate.

4. The turnover number of erythrocyte cholinesterase determined in this way has been found to

vary, not only among species, but also among individuals of the same species.

5. It is concluded that the inhibitors are not so highly specific as was previously believed. The variations in apparent turnover number are probably due to the presence in the tissue extracts of varying amounts of other substances competing with cholinesterase for the inhibitor.

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REFERENCES

Aldridge, W. N. (1950). *Biochem. J.* **46**, 451.
Aldridge, W. N., Berry, W. K. & Davies, D. R. (1949). *Nature, Lond.*, **164**, 925.
Augustinsson, C. B. & Nachmansohn, D. (1949). *J. biol. Chem.* **179**, 543.
Boursnell, J. C. & Webb, E. C. (1949). *Nature, Lond.*, **164**, 875.
Chapman, N. B. & Saunders, B. C. (1948). *J. chem. Soc.* p. 1010.
Davies, D. R. & Smellie, R. M. S. (1949). Unpublished observations.
Easson, L. H. & Stedman, E. (1936). *Proc. roy. Soc.* B, **121**, 142.
Goldstein, A. (1944). *J. gen. Physiol.* **27**, 529.
Jansen, E. F., Nutting, M-D. F. & Balls, A. K. (1948). *J. biol. Chem.* **175**, 975.
Jansen, E. F., Nutting, M-D. F. & Balls, A. K. (1949). *J. biol. Chem.* **179**, 201.
Mackworth, J. F. & Webb, E. C. (1948). *Biochem. J.* **42**, 91.
Mazur, A. (1946). *J. biol. Chem.* **164**, 271.
Mazur, A. & Bodansky, O. (1946). *J. biol. Chem.* **163**, 261.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Mounter, L. A. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 525.
Nachmansohn, D., Rothenberg, M. A. & Feld, E. (1948). *J. biol. Chem.* **174**, 247.
Roepke, M. H. (1937). *J. Pharmacol.* **59**, 264.
Rothenberg, M. A. & Nachmansohn, D. (1947). *J. biol. Chem.* **168**, 223.
Snedecor, G. W. (1946). *Statistical Methods*, p. 105, § 6·3. Iowa: State College Press.
Straus, O. H. & Goldstein, A. (1943). *J. gen. Physiol.* **26**, 559.
Strelitz, F. (1944). *Biochem. J.* **38**, 86.
Webb, E. C. (1948). *Biochem. J.* **42**, 96.

The Preparation and Properties of β-Glucuronidase

2. CENTRIFUGAL FRACTIONATION OF WATER HOMOGENATES AND THE NATURE OF THE SEDIMENTABLE FRACTION

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In the first paper in this series (Kerr & Levvy, 1951) it was shown that buffering a water homogenate of mouse liver, kidney or uterus to a slightly acid pH led to fractionation of the β-glucuronidase activity on subsequent centrifuging at low speeds. Changes in the glucuronidase activity of these organs with the state of proliferation (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949) were seen to be confined to the non-sedimentable fraction. In normal adult liver the sedimentable fraction accounted for nearly half the total glucuronidase activity in the buffered water homogenate. As described below, identical fractionation of mouse-liver glucuronidase in untreated water homogenates can be achieved on the high-speed centrifuge. The partition of the enzyme has been correlated with microscopic observations in the homogenate, and methods for bringing the sedimentable fraction into solution have been studied.

METHODS

Preparation and fractionation of the enzyme. Mouse liver was ground with water for 1 min. in a glass homogenizer. We have found Veridia precision bore glass tubing (Chance Bros. Ltd.) convenient for the manufacture of homogenizers. After dilution of the homogenate to a convenient volume, samples were taken for measurement of the total enzyme activity. Other samples were fractionated by one or both of the following methods: (*a*) After pH adjustment, the preparation was centrifuged on the M.S.E. (Measuring and Scientific Equipment Ltd., London, S.W. 1) Minor at 1500 *g* for 15 min. If, as was usually the case, acetate buffer (pH 5·2) was used to adjust the pH, the terms 'acetate-soluble' and 'acetate-insoluble' are sometimes used for the enzyme in the supernatant and sediment, respectively. (*b*) The preparation was centrifuged without pH adjustment on the M.S.E. Major with Superspeed Attachment. This was usually done at 20,000–25,000 *g* for 15 min., yielding 'soluble' enzyme in the 'high-speed supernatant', and 'insoluble' enzyme in the sediment. The Superspeed centrifuge head was chilled before use.

Tissue concentrations in preparations taken for centrifuging by either method varied from 2·5 to 10% (w/v). This factor was without influence on the distribution of enzyme activity between the two fractions.

In measuring the partition of enzyme activity, the figure for the sediment was found by difference from the figures for total and unsedimented enzyme. To determine the latter, a sample of the supernatant was withdrawn directly from the centrifuged preparation. For calculation, it was assumed that the unsedimented enzyme was uniformly distributed in the supernatant and debris. This assumption was found to be justified when the results were compared with those obtained by direct assay of both fractions after separation by method (*a*) (Table 1). For this purpose the supernatant was decanted and the sediment washed once on the centrifuge with one-tenth the original volume of fluid and then resuspended for assay with the aid of the glass homogenizer. The wash fluid was added to the supernatant fraction. The sediment separated by method (*b*) was seldom sufficiently well packed to permit complete decanting of the supernatant and thus direct measurement of the distribution of the enzyme. Nevertheless, even though separation was incomplete, the results for this method agreed with those for method (*a*) (Table 1) in showing that the sum of the figures for supernatant and sediment agreed with the figure for the total glucuronidase activity.

Enzyme assay. This was carried out as described by Kerr & Levvy (1951). All enzyme preparations were diluted to a final volume of 20–40 ml./g. moist tissue, and 0·5 ml. was transferred to the tube containing phenolphthalein glucuronide and acetate buffer, pH 5·2. As noted by Kerr & Levvy (1951), incomplete dilution of preparations containing insoluble enzyme may lead to low results in the assay. Results are expressed as glucuronidase units (G.U.), where 1 G.U. liberates 1 μg. phenolphthalein from 0·00125 M-phenolphthalein glucuronide in 1 hr. at 37° and pH 5·2.

Microscopy. Wet preparations were examined with the Cooke, Troughton and Simms Ltd. phase-contrast microscope, which is fitted with a phase plate giving 20% transmission and a retardation of $\lambda/4$. Using an oil immersion lens, N.A. 1·3, and ×15 compensating eyepieces, a final magnification of ×2000 was attained with almost theoretical resolution (0·2 μ.: cf. Discombe, 1950).

EXPERIMENTAL AND RESULTS

Identity of fractionation in buffered and unbuffered water homogenates

In studying the fractionation of glucuronidase at ordinary centrifuge speeds, Kerr & Levvy (1951) usually adjusted the pH to 5·2 with acetate or citrate buffer. Of these buffers, acetate was the more convenient to use for purposes of comparison with the fractionation of untreated homogenates on the high-speed centrifuge, since the partition of enzyme activity is less dependent on the pH and buffer concentration with acetate than with citrate.

Centrifuging an untreated homogenate of adult mouse liver at increasing speed caused a progressive fall in the activity of the supernatant (Fig. 1) and the sedimentation of an increasing proportion of that enzyme fraction which was acetate-insoluble, as was shown by subsequent fractionation of the various supernatants in acetate buffer. After centrifuging the unbuffered homogenate at 25,000 *g* for 15 min., none of the residual supernatant activity was precipitated on buffering with acetate.

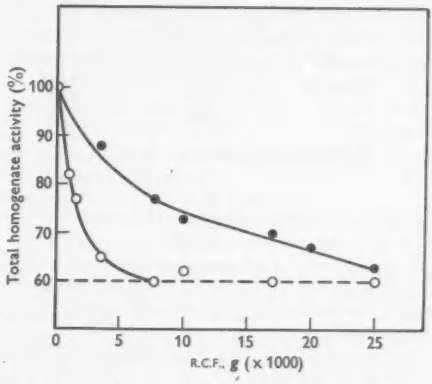

Fig. 1. Glucuronidase activity of the supernatant from an unbuffered water homogenate of adult mouse liver centrifuged at varying speeds for 15 min. Total residual activities expressed as percentages of the initial homogenate activity. ●, untreated homogenate; ○, homogenate made 0·1 M with respect to NaCl; – – –, acetate-soluble fraction in homogenate.

At moderate relative centrifugal force (R.C.F.) values, sedimentation of the insoluble enzyme in an unbuffered homogenate could be increased by lengthening the period of centrifuging. At 20,000–25,000 *g* (the maximum attainable with our equipment), however, no further sedimentation occurred on centrifuging for 60 instead of 15 min. The heating of the preparation during centrifuging had no effect on the overall enzyme activity, nor on the partition of the enzyme.

Table 2 gives average figures for the fractionation of adult and infant mouse-liver glucuronidase on the high-speed centrifuge (20,000–25,000 *g* for 15 min.), carried out in parallel with fractionation by acetate buffer. There was excellent agreement between the two methods of fractionation and, as noted by Kerr & Levvy (1951), the fall in glucuronidase activity in passing from the infant to the adult tissue (Levvy *et al.* 1948) was confined to the soluble fraction.

In other experiments with infant and adult liver it was found that no material, active or inactive, could be brought down in the supernatant from buffered homogenates separated at 1500 *g* on subsequent centrifuging at 25,000 *g*. Unbuffered high-speed supernatants, on the other hand, although devoid of acetate-insoluble enzyme, did contain inactive protein precipitable with acetate buffer, pH 5·2.

Table 1. *Comparison of alternative procedures for studying the fractionation of mouse-liver glucuronidase*

(Homogenate of adult liver made 0·1 M with respect to acetate buffer, pH 5·2, and centrifuged at 1500 *g* for 15 min. In columns I, the washed sediment was assayed for insoluble enzyme activity. In columns II, the activity of this fraction was found by difference as described in the text.)

	Glucuronidase units/g. liver				
		I		II	
Exp. no.	Total	Supernatant	Sediment	Supernatant	Sediment (calc.)
1	3200	1740	1480	1780	1420
2	3100	1620	1520	1620	1480
3	2680	1500	1200	1520	1160

Table 2. *Fractionation of mouse-liver glucuronidase on the high speed centrifuge, and on the low speed centrifuge after addition of acetate buffer*, pH 5·2

(Values shown as mean ± S.E. followed in parenthesis by the number of experiments.)

	Glucuronidase units/g. liver			
	High-speed centrifuge		Acetate treatment	
Description	Soluble	Insoluble	Soluble	Insoluble
Normal adult	1707 ± 46 (12)	1161 ± 48 (12)	1625 ± 37 (12)	1243 ± 48 (12)
Infant (4–6 days old)	3003 ± 108 (7)	1173 ± 88 (7)	2943 ± 120 (7)	1262 ± 98 (7)

Microscopic observations

Under the phase microscope, a water homogenate of mouse liver was seen to be composed in the main of discrete spherical granules, ranging continuously in size from 5 μ. down to the limit of visibility. The smaller were by far the more numerous. A few intact or partially disintegrated cells, some nuclei and a variable number of fat globules were also present. Whilst we do not propose to discuss the fine structure of these components in detail, it should be noted that, with the exception of the fat globules, they all showed signs of pronounced osmotic swelling. Some of these effects have been observed by Zollinger (1948) under the phase microscope, by Opie (1949) in fixed stained preparations, and by Dalton, Kahler, Kelly, Lloyd & Striebich (1949) under the electron microscope. As shown below, any arbitrary subdivision of the continuous range of granules visible under the phase microscope into such groups as 'mitochondrial' or 'large' on the one hand, and 'small' on the other hand, has no significance in the present connexion. In the following pages, therefore, the term granule is used for all cytoplasmic particles visible under the microscope, with the exception of the fat globules.

Centrifuging untreated homogenates of adult or infant mouse liver at increasing speeds caused a continuous fall in the number of granules in suspension, corresponding to the fall in supernatant glucuronidase activity illustrated in Fig. 1. Complete sedimentation of insoluble enzyme was not achieved until all but a very few of the smallest visible granules had been removed from the supernatant by centrifuging at 25,000 *g* for 15 min. Addition of acetate buffer, pH 5·2, to a water homogenate of mouse liver gave rise to an optically clear supernatant after centrifuging at 1500 *g* for 15 min. Microscopically, the sediment consisted of large clumps of material formed by the agglutination of all the visible components of the homogenate, together with an amorphous protein precipitate. The precipitation of inactive soluble protein and the agglutination of the granules were observed separately by adding acetate buffer to an unbuffered high-speed supernatant, and to an aqueous suspension of the corresponding sediment, respectively.

It is clear that the sedimentable enzyme in a water homogenate of mouse liver is associated with granules of all sizes, and that the ease with which this enzyme fraction may be sedimented after the addition of acetate buffer is a result of the agglutination of the granules. The acetate-soluble enzyme, on the other hand, is in true solution, so far as we could judge microscopically and from its behaviour on the high-speed centrifuge.

The effects of pH *and electrolyte concentration*

Kerr & Levvy (1951) found that the partition of the enzyme by acetate buffer at 1500 *g* was independent of the pH over the range 5·0–5·6 (0·1 M-buffer), and of the buffer concentration over the range 0·5–0·025 M (pH 5·2). More extensive investigation of the effect of pH in 0·1 M-acetate buffer has shown that the fractionation of the enzyme at 1500 *g* did not change between the pH limits 4 and 6. Within this range, agglutination of the granules was pronounced. Incomplete sedimentation of the insoluble enzyme on the low-speed centrifuge outside the pH limits stated was correlated with incomplete

agglutination of the granules. In such cases, subsequent high-speed centrifuging brought down the residual granules and associated insoluble enzyme.

At pH 6·8, the natural pH of the homogenate, the buffered preparation showed slight agglutination of the granules, and 20 % of the activity sedimented at 1500 *g*. This contrasted with a figure of 10 % in the untreated homogenate. At this pH the acetate buffer acted purely as an electrolyte, since adding an equivalent concentration of sodium chloride produced the same effect. The action of this electrolyte is shown in Fig. 1. Making an unbuffered water homogenate 0·1 M with respect to sodium chloride led to much easier sedimentation of the granules and associated glucuronidase activity.

With citrate, as opposed to acetate, buffer, Kerr & Levvy (1951) found the upper pH limit for complete sedimentation of the insoluble enzyme at 1500 *g* to be 5·2. Microscopic studies have shown that above this pH in citrate buffer (0·033 M) agglutination of the granules was incomplete.

In an unbuffered homogenate, pH adjustment to 5·0 with hydrochloric or acetic acid gave the usual fractionation of the liver enzyme at 1500 *g*. At slightly higher pH values, however, the use of acid alone led not only to agglutination of the granules, but also to precipitation of part of the soluble enzyme. This can be seen from Table 3 by comparing figures for the activity in the supernatant from an acid-adjusted homogenate with the corresponding figures for a buffered homogenate. Sedimentation of enzyme activity over and above that associated with the granules was at an optimum at pH 5·5. About a quarter of the soluble enzyme was precipitated by acid at this pH. Addition of 0·1 M-sodium chloride to the homogenate prevented the precipitation of soluble enzyme, and acidifying then gave rise to the usual distribution of the enzyme up to the pH limit for complete agglutination. It would appear that the partition of glucuronidase activity in centrifuged liver homogenates buffered with acetate is not entirely a question of pH, but is in part determined by the electrolyte concentration. On repeating the experiments of Kerr & Levvy (1951) we have formed the impression that a concentration of 0·025 M-acetate is barely high enough to ensure that the soluble enzyme is always kept entirely in solution at pH 5·2.

In other experiments it was found that part of the activity in preparations containing only soluble enzyme (high-speed supernatants) could be precipitated by adding acid. The optimum again appeared to be at pH 5·5, at which a quarter to a third of the enzyme sedimented. Centrifuging at 25,000 instead of 1500 *g* did not increase the amount of enzyme precipitated, nor did prolonged dialysis of the preparation prior to acidification.

Precipitation of soluble glucuronidase by acid was readily reversed by warming the preparation. After 15 min. at 37°, only the granular enzyme sedimented from liver homogenates adjusted to pH 5·5 with hydrochloric or acetic acid. The question of precipitation of soluble enzyme does not arise in other sections of this paper.

Measures bringing the granular enzyme into solution

Incubation at 37°. Kerr & Levvy (1951) showed that incubation in acetate buffer, pH 5·2, for 4 hr. converted the granular enzyme into a soluble form. This change did not occur in citrate buffer at the same pH. It has now been found that conversion of granular into soluble enzyme does not occur in unbuffered homogenates after incubation for 24 hr. at the natural pH (6·8), nor after pH adjustment to 5·2 with hydrochloric or acetic acid and incubation for 6 hr. There was no fall in the total activity of the incubated preparations in these experiments.

The complete conversion of granular enzyme into soluble enzyme after 4 hr. at 37° and pH 5·2 in acetate buffer has been confirmed with the high-speed centrifuge. Inspection of the incubated preparations under the microscope revealed no dispersion of the agglutinated granules and surprisingly little change in their general appearance.

Table 3. *Precipitation of the soluble enzyme by acid*

(The pH of the adult liver homogenate was adjusted by the method shown. After centrifuging at 1500 *g* for 15 min., assays were done at pH 5·2 in acetate buffer, and the results are expressed as percentages of the initial homogenate activity.)

pH of fractionation	Residual supernatant activity (%)				
	0·1 M-Acetate buffer	HCl	Acetic acid	0·1 M-NaCl + HCl	Agglutination*
4·6	59	60	57	57	+ + + +
5·0	58	57	54	55	+ + + +
5·3	57	49	47	55	+ + + +
5·5	57	43	41	55	+ + + +
5·8	58	52	56	55	+ + + +
6·1	64	64	67	60	+ +

* Arbitrary assessment of degree of agglutination.

Acetate would thus appear to have extracted the enzyme from the granules.

The tissue disintegrator. Shaking a homogenate of mouse liver with Ballotini, Grade 12 (Chance Bros. Ltd.) in the Mickle Tissue Disintegrator (Mickle, 1948) for 30 min. caused almost complete conversion of the granular enzyme into a soluble form, without appreciable inactivation. At the end of this treatment all the components of the homogenate were seen to have undergone disintegration, with complete loss of structure. On the high-speed centrifuge, part of the amorphous debris sedimented and part moved centripetally, leaving a clear intermediate layer; acetate buffer, pH 5·2, caused agglutination and ready sedimentation of the debris, leaving 94% of the enzyme activity in solution. Prior treatment of a homogenate with acetate buffer did not lessen the disruption of the granules and the release of enzyme to the solution on subsequent treatment in the disintegrator.

Freezing and thawing. Table 4 shows that repeated freezing in a mixture of solid carbon dioxide and acetone, and thawing at 37°, caused release of glucuronidase activity from the granules in a liver homogenate to the solution. From microscopic observations, this treatment appeared to result in disruption of the granules.

Table 4. *Release of glucuronidase activity from the granules by repeated freezing and thawing*

(Enzyme fractionated with acetate buffer, pH 5·2, and on the high-speed centrifuge.)

No. of freezings and thawings	Glucuronidase units/g. liver		
	Total in homogenate	Acetate-soluble enzyme	High-speed supernatant
0	3100	1680	1700
1	—	2100	—
4	—	2300	—
8	3100	2600	2600

Fishman, Springer & Brunetti (1948), in studying the glucuronidase activity of white blood cells, used this method and found that 'the disruption of the cell membranes by water laking did not release as much enzymic activity as appeared in the lysate after the intermittent freezing-thawing procedure'. It appears possible from this that part of the enzyme in the white blood cell, as in the liver cell, is closely associated with cytoplasmic particles, but the original paper does not give sufficient experimental detail to permit any conclusion on this point.

Treatment with acetone. Homogenizing in acetone converted a large part of the granular enzyme into the water-soluble form. This is illustrated in Table 5. In Exp. 1, part of the liver was homogenized in acetone (about 5 ml./0·5 g. tissue) and centrifuged. The acetone was poured off and the last traces removed by blowing air into the centrifuge tube. When the dry powder was resuspended by homogenizing in water, 85% of the total glucuronidase activity was in the soluble fraction. The corresponding figure for another piece of the same liver homogenized directly in water was 60%. Exp. 1 also shows that the treatment of liver with acetone resulted in a 6% drop in overall activity, whether through enzyme inactivation or a loss to the acetone layer it was impossible to decide. Microscopic examination of the acetone powder after resuspension in water showed that the majority of the granules had disintegrated. The remainder were shrunken, opaque and irregular in outline.

In Exp. 2, in Table 5, the liver was homogenized in water and the homogenate divided into two portions. Both samples were made 0·1 M with respect to acetate buffer, pH 5·2, and centrifuged. The acetate-insoluble material was washed with 0·1 M-buffer, and rehomogenized in water and acetone respectively. The acetone homogenate was treated as above, and 79% of the granular enzyme was finally obtained in a water-soluble form. From the control it could be seen that rehomogenizing the acetate-precipitated material in water converted

Table 5. *The effect of acetone on the distribution of glucuronidase activity in adult mouse liver*

(The dry powder obtained from the acetone-treated tissue or tissue preparation was resuspended in the aqueous medium shown prior to fractionation with acetate buffer or on the high-speed centrifuge.)

Exp. no.	Starting material	Homogenizing fluid	Suspension fluid for fractionation	Glucuronidase units/g. liver		
				Total	Acetate-soluble enzyme	High-speed supernatant
1	Fresh liver	Water	—	2880	1720	—
		Acetone	Water	2720	2320	2320
2	Precipitate of acetate-insoluble material	Water	—	1720	380	—
		Acetone	Water	1680	1320	1350
3	Fresh liver	Acetone	Water	2800	2300	2350
		Acetone	0·1 M-NaCl	2800	1960	—
		Acetone	Acetate buffer	2800	1680	—

22% of the granular enzyme into the soluble form. The loss in enzyme activity resulting from the acetone treatment of the acetate-precipitated material was only 2%.

In the third experiment in Table 5, the acetone powder from whole liver was rehomogenized in various aqueous media. In water, as before, more than 80% of the enzyme activity was found in the soluble fraction. The use of 0·1 M-acetate buffer, pH 5·2, as suspension fluid, however, resulted in a similar partition of the activity to that seen in a water homogenate of fresh liver (cf. Table 2). The use of 0·1 M-sodium chloride at this stage resulted in a distribution of the enzyme which was intermediate in character between those obtained with water and with acetate buffer. It would appear from this experiment that agglutination of the granules during the resuspension of the acetone powder in an aqueous medium prevents the escape of their activity to the solution.

Acetone has been used in the large-scale preparation of glucuronidase from ox spleen (Fishman, 1939; Graham, 1946). Fishman (1939) precipitated the enzyme from an aqueous extract of the minced tissue with acetone, but Graham (1946) found that he obtained a greater final yield of the enzyme by treating the tissue mince directly with acetone and extracting the dry powder with water. It seems possible that the better yield in Graham's procedure was due as much to the conversion of granular enzyme into a water-soluble form as to the avoidance of losses originally suggested.

Treatment with surface-active agents. In view of their well known cytolytic actions (see, for example, Glassman, 1950), the effect of surface-active agents on the granules in water homogenates of mouse liver were studied. The ionic surface-active agents, in addition to their cytolytic properties, combine with proteins in general (Putnam, 1948). Depending upon the conditions, this reaction may result in precipitation, denaturation or catalysed hydrolysis of the protein, and enzymes may lose their activities.

Teepol XL (British Drug Houses Ltd.), a typical anionic surface-active agent, is a mixture of sodium salts of secondary alkyl sulphates, containing from eight to seventeen carbon atoms, and with an average mol.wt. of 304 (Shell Chemicals Ltd., private communication). This substance was added as a neutral solution to homogenates in which the final tissue concentration was 2·5% (w/v), and the preparations were fractionated, either on the high-speed centrifuge or by treatment with acetate buffer, as rapidly as possible (in less than 30 min.). Assay followed immediately. As can be seen from Table 6, low concentrations of Teepol caused an increase in the proportion of the enzyme activity which was soluble. Almost all the enzyme was soluble in 0·5% (w/v) Teepol, at which concentration most of the particulate tissue material was no longer visible under the microscope. Remaining granules showed greatly reduced contrast. This concentration of Teepol also caused a slight increase in the total homogenate activity, due no doubt to the greater dispersion of the enzyme (cf. Kerr & Levvy, 1951). Release of the granular enzyme to the solution by Teepol depended not only on the concentration, but also on the period of contact. Thus, with a Teepol concentration of 0·05% (w/v), Table 6 shows that 77% of the total enzyme activity was in solution. The corresponding figure, when fractionation was delayed by 2 hr. after adding the surface-active agent to the homogenate, was 97%.

Table 6. *The action of surface-active agents on water homogenates of adult mouse liver*

(Surface-active agent added in concentrations shown to samples of a homogenate in which the final tissue concentration was 2·5%. Enzyme fractionated as quickly as possible with acetate buffer, pH 5·2, and on the high-speed centrifuge. All figures obtained with the same liver preparation.)

Concentration of surface-active agent* (% w/v)	Glucuronidase units/g. liver: Total in homogenate	Acetate-soluble enzyme	High-speed supernatant
Control	2830	1710	1760
Teepol XL			
0·05	2800	2160	—
0·15	2880	2560	2640
0·50	2960	2800	2800
1·00	2640	480	2160
1·25	720	160	—†
1·50	120	0	—†
2·00	0	0	—
Triton X-100			
0·01	2960	2000	—
0·02	2960	2320	2240
0·04	3040	2720	—
0·12	3040	3040	3040
0·25	3040	3120	—
0·50	2960	2960	3040
2·00	2880	2960	—
Triton A-20			
0·10	2880	1840	1920
0·50	2880	1840	—
1·00	2880	1920	1920
2·00	2880	1760	—

* These concentrations underwent an eightfold dilution when the enzyme was transferred to the assay tubes.

† In other experiments with this Teepol concentration, figures for the activity in the high-speed supernatant were intermediate between those for total homogenate and acetate-soluble enzyme.

At Teepol concentrations of 1% (w/v) and more, inhibition of glucuronidase became evident. This effect was more marked in the acetate-soluble enzyme preparation than in the high-speed supernatant, and in both than in the unfractionated

homogenate; that is inhibition was most pronounced when the protein content of the preparation was least. Inhibition of glucuronidase by Teepol and the protective effect of inactive protein is dealt with more fully in the next section.

Glassman (1950) has shown that certain non-ionic surface-active agents are non-haemolytic. Two of the non-ionic agents he studied, the non-haemolytic Triton A-20 and the haemolytic Triton X-100 (formerly labelled Triton N-100 and listed thus in Glassman's paper), were obtained from the Rohm and Haas Company, Philadelphia. Experiments similar to those carried out with Teepol showed that the actions of the two Triton samples on the liver granules paralleled their haemolytic properties (Table 6). Triton A-20 had no effect, whatsoever, whilst Triton X-100 in a concentration of 0·12% (w/v) caused complete solution of the granular enzyme and the disappearance of visible particles from the homogenate. In the lower concentrations, the effect of Triton X-100 depended upon the period of contact. When fractionation was delayed by 2 hr. the soluble enzyme fraction in a homogenate containing 0·02% Triton X-100 rose from 78 to 84%. Greater overall homogenate activity due to dispersion of the enzyme was also seen with this agent. Since the non-ionic surface-active agents do not react with proteins in general, it was not surprising to find that neither Triton X-100 nor Triton A-20 caused any appreciable fall in glucuronidase activity in the concentrations studied.

Kerr & Levvy (1951) obtained false low results for the total glucuronidase activity of mouse-liver homogenates when the assay was carried out at too high a tissue concentration. The true activity was displayed at all tissue concentrations after complete conversion of the enzyme into a soluble form by incubating in acetate buffer. The slight activation of glucuronidase in liver homogenates sometimes observed with the surface-active agents, Teepol XL and Triton X-100, probably indicated greater dispersion of the enzyme than could be achieved by dilution alone (cf. 'Enzyme assay').

Inhibition of glucuronidase by Teepol XL

The inhibition of mouse-liver glucuronidase by Teepol XL noted above (Table 6) was further investigated in experiments in which changes in the partition of the enzyme were ignored. The first series of experiments dealt with the variation in inhibition with Teepol concentration and with the amount of inactive protein present (Fig. 2). The enzyme preparation consisted of a water homogenate of adult mouse liver in which the tissue concentration was 3·3% (w/v), or of the supernatant obtained by fractionating the homogenate in acetate buffer. For assay, the incubation mixture contained 0·5 ml. enzyme, 0·5 ml. substrate solution, and 3 ml. 0·1 M-acetate buffer (pH 5·2) to which the Teepol was added. In some experiments, 0·25 ml. buffer was replaced by the same volume of boiled homogenate. The liberation of phenolphthalein after 1 hr. at 37° in presence of Teepol was compared with that seen in its absence.

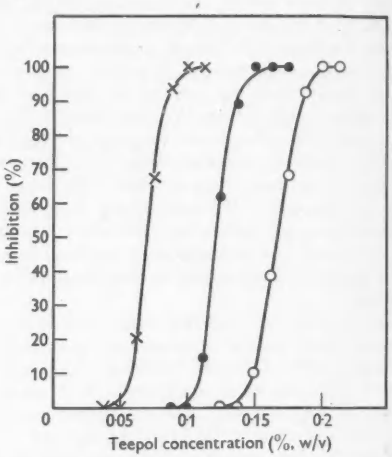

Fig. 2. Inhibition of glucuronidase by Teepol XL and protection by inactive protein. Substrate, 0·00125 M-phenolphthalein glucuronide in 0·1 M-acetate buffer, pH 5·2. Teepol added to assay tubes in concentrations shown. ○—○, water homogenate of mouse liver; ×—×, acetate-soluble enzyme separated from above; ●—●, acetate-soluble enzyme plus inactive protein.

Inhibition of glucuronidase occurred at much lower Teepol concentrations in the preparation of acetate-soluble enzyme than in the entire homogenate. The former preparation contained much less inactive protein than the latter (see above). Addition of inactive protein (boiled homogenate) to the acetate-soluble enzyme protected against concentrations of Teepol which otherwise were completely inhibitory. Rabinovitz & Boyer (1950) used inactive protein to protect succinic oxidase against inhibition by sodium dodecyl sulphate. Different livers varied slightly in the degree of inhibition of glucuronidase produced by Teepol under fixed conditions. This was no doubt due to variations in the protein content of the wet tissue.

Inhibition of glucuronidase by Teepol XL was non-competitive. This was shown by the fact that the percentage inhibition of the hydrolysis of phenolphthalein glucuronide by acetate-soluble mouse liver glucuronidase in the presence of 0·06% (w/v) Teepol did not vary over a range of substrate concentrations, the lowest of which (0·0003 M) did not completely saturate the enzyme, whilst the highest (0·005 M) was itself inhibitory.

Delay in adding inactive protein made it progressively less effective in reversing inhibition by Teepol. This is shown in Fig. 3. The enzyme preparation was the supernatant from a 3·3% (w/v) homogenate fractionated with acetate buffer. After varying periods of contact with the enzyme at room temperature, the action of the surface-active agent was arrested by transferring the preparation to assay tubes containing excess inactive protein (0·25 ml. boiled homogenate). The rate at which

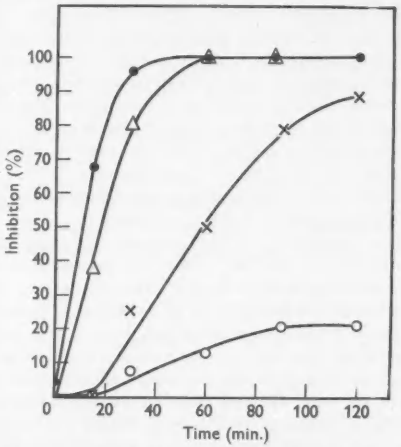

Fig. 3. Irreversible inhibition of glucuronidase after varying periods of contact with Teepol XL at room temperature. Teepol added in concentrations shown to a preparation of acetate-soluble enzyme. Samples transferred at each time interval to assay tubes containing excess inactive protein. ○—○, 0·5% Teepol; ×—×, 0·55% Teepol; ●—●, 0·60% Teepol; △—△, 0·5% Teepol with enzyme diluted to 75% of its former concentration.

irreversible inactivation of glucuronidase took place depended upon the relative concentrations of enzyme and Teepol. A decrease in the former accelerated inactivation in the same way as an increase in the latter. Inactivation of the enzyme was probably a result of denaturation similar to that described for other proteins treated with ionic surface-active agents (Putnam, 1948).

The question arose whether the inhibition of glucuronidase by Teepol shown in Fig. 2 and Table 6 was entirely due to progressive denaturation of the enzyme during the incubation with substrate, or whether in addition to denaturation there was immediate reversible inhibition of the enzyme by the surface-active agent. To answer this question, velocity curves for the hydrolysis of phenolphthalein glucuronide were drawn up as shown in Fig. 4. Samples were withdrawn for the measurement of free phenolphthalein at 15 min. intervals after the addition of acetate-soluble enzyme to various incubation mixtures.

In each of the two experiments (*a* and *b* in Fig. 4), five flasks were set up containing phenolphthalein glucuronide in acetate buffer, pH 5·2. In four flasks the required amount of Teepol was incorporated in the buffer, and to three of these excess inactive protein (boiled homogenate) was added at 0,

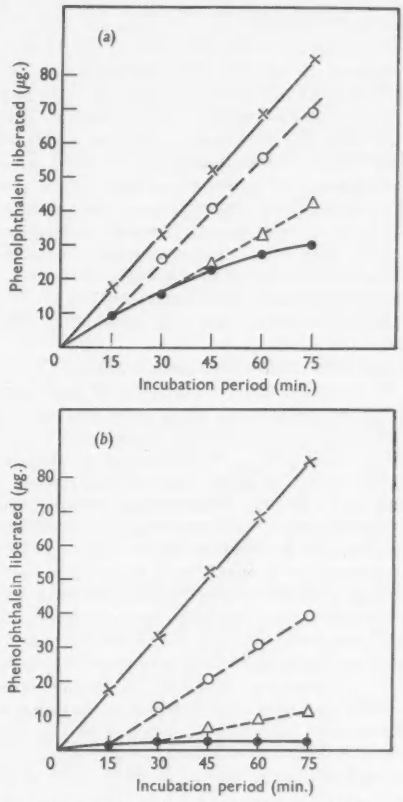

Fig. 4. The rate of hydrolysis of phenolphthalein glucuronide by glucuronidase in the presence of (*a*) 0·1% and (*b*) 0·12% Teepol XL, and the effect of adding excess inactive protein. ×—×, control (flask 1); ●—●, Teepol alone (flask 2); ○– – –○, Teepol, with addition of inactive protein after 15 min. incubation (flask 4); △– – –△, Teepol, with addition of protein after 30 min. (flask 5).

15 and 30 min. after the enzyme, respectively. In the other two flasks, inactive protein was replaced by an equal volume of water added with the enzyme. The contents of the flasks may be summarized as follows:

(1) Enzyme + substrate + buffer + water.
(2) Enzyme + substrate + buffer + Teepol + water.
(3) Enzyme + substrate + buffer + Teepol + inactive protein at 0 min.

(4) Enzyme + substrate + buffer + Teepol + inactive protein at 15 min.
(5) Enzyme + substrate + buffer + Teepol + inactive protein at 30 min.

The rate of hydrolysis after any time interval is given in Fig. 4 by the slope of the line or of the tangent to the curve at that point. In both experiments, the results for flask 3, in which the enzyme was protected against Teepol from the start, were identical with those for the control (flask 1), and are not shown. In absence of added protein, Teepol (flask 2) caused a progressive falling off in the rate of hydrolysis, which rapidly reached zero in 0·12% Teepol (Fig. 4*b*). Addition of inactive protein after 15 or 30 min. (flasks 4 and 5) not only arrested denaturation, but caused an increase in the rate of hydrolysis in both experiments, showing that inhibition of glucuronidase by Teepol is indeed partly reversible. Protein caused a smaller increase in reaction velocity after 30 than after 15 min., indicating that denaturation advanced at the expense of reversible inhibition, and was probably a further stage in the same process.

In addition to the effects already described, Teepol XL in low concentration precipitated inactive protein from preparations of the acetate-soluble enzyme at pH 5·2. With increasing concentration of the surface-active agent, the precipitate started to redissolve, and this point was reached before any change in the activity of the enzyme became evident. No precipitation of active protein was observed at any Teepol concentration under these conditions. Precipitation of a protein by a given ionic surface-active agent depends upon the pH of the medium and the isoelectric point of the protein (Putnam, 1948).

The complete recovery of glucuronidase activity caused by immediate addition of inactive protein to enzyme preparations inhibited by Teepol supports the view that the initial interaction between ionic surface-active agents and proteins in general, as distinct from the subsequent denaturation, is fully reversible (Putnam, 1948).

DISCUSSION

It is now clear that the fractionation of glucuronidase activity produced in water homogenates of mouse liver by buffering to a slightly acid pH (Kerr & Levvy, 1951) corresponds to a real difference in the location of the two fractions in the untreated homogenate. This has been demonstrated with the aid of the microscope and the high-speed centrifuge. One fraction appeared to be in true solution whilst the other was associated with the cytoplasmic granules. The ready sedimentation of the latter enzyme fraction observed in buffered preparations was due to agglutination of the granules. Agglutination by acetate buffer was analysed into two components, the effect of pH and the effect of the electrolyte concentration. The former factor was the more important for complete agglutination, but a relatively high electrolyte concentration was required to prevent partial precipitation of the soluble enzyme in the pH region for agglutination. Glucuronidase activity was found in granules of all sizes. This was in contrast to succinic oxidase activity, which is shown by only the larger granules in tissue homogenates (Schneider & Hogeboom, 1950). With this complex enzyme system only comparatively large particles can be expected to contain all the essential components. This consideration does not apply to a simple hydrolytic enzyme such as glucuronidase. However, the use of water as the homogenizing fluid caused pronounced osmotic swelling of the nuclei and other cell particles, leading sometimes to disruption. The use of a fluid which tends to preserve these bodies, such as isotonic sodium chloride solution, may be expected to lead to quite a different fractionation of glucuronidase activity on centrifuging the homogenate. This point is at present under investigation.

The conversion of the granular enzyme into a soluble form observed when the homogenate was incubated in acetate buffer (Kerr & Levvy, 1951) was not a simple autolytic process, but appeared to be fairly specific for acetate ion. Microscopic studies suggested that the enzyme was extracted from the granules during the incubation in acetate. Of other methods for bringing this enzyme fraction into solution, shaking with Ballotini and freezing and thawing caused disintegration of the granules. The action of acetone suggested that the granules are normally surrounded by a protective fatty layer, removal of which permits the escape of water-soluble protein. The non-ionic surface-active agent, Triton X-100, may have acted like acetone in this respect, and the anionic Teepol XL may have produced its effect in the same way or by direct combination with protein. It is tentatively suggested that acetate behaved as a feeble surface-active agent in causing release of the granular enzyme.

Inhibition of glucuronidase by Teepol XL, but not by Triton X-100, is in accordance with the general finding that ionic surface-active agents react with proteins whilst non-ionic do not. It is interesting to contrast the reaction of this simple enzyme towards agents of this type with that of liver succinic oxidase which is inhibited by both ionic and non-ionic surface-active agents (Hockenhull, 1948). It is reasonable to suppose that dispersion of the individual components of a complex system by a non-ionic agent could itself lead to inhibition.

SUMMARY

1. About 40% of the glucuronidase activity in a water homogenate of adult mouse liver was sedimented by centrifuging at 25,000 *g* for 15 min. This corresponded to the removal from suspension of

almost all the cytoplasmic granules visible under the phase microscope. Glucuronidase activity was distributed over granules of all sizes.

2. Making the preparation 0·1 M with respect to sodium chloride led to slight agglutination and much easier sedimentation of the granules and the associated enzyme activity.

3. Buffering the preparation to pH 4–6 with acetate led to pronounced agglutination of the granules, which then sedimented after 15 min. at 1500 *g*. Adjusting the pH to within these limits with acid alone led not only to agglutination of the granules, but to precipitation of part of the water-soluble enzyme. The latter effect was overcome by adding electrolyte, as in buffered preparations, or by warming.

4. Incubation in acetate buffer, pH 5·2, led to extraction of the enzyme from the granules. This did not occur on incubating an untreated homogenate, or after pH adjustment with acid alone.

5. Shaking a homogenate in a tissue disintegrator, or repeated freezing and thawing, caused disintegration of the granules and release of the associated enzyme to the solution. Homogenizing the tissue in acetone followed by resuspension in water had the same effect, but release of the enzyme was not seen when the acetone-dried powder was resuspended in acetate buffer.

6. Treatment with the surface-active agents, Teepol XL and Triton X-100, brought the granular enzyme into solution with disappearance of particulate matter from the preparation.

7. Glucuronidase was inhibited non-competitively by the anionic Teepol XL, but not by the non-ionic Triton X-100. Inhibition by Teepol was at first reversed by adding inactive protein, but later became irreversible.

The authors wish to express their gratitude to their colleague, Mr F. Baker, for instruction in the use of the phase microscope, and to the Rohm and Haas Company for gifts of Triton A-20 and X-100. Contact with this firm was made through the courtesy of Dr H. N. Glassman. One of us (P.G.W.) is in receipt of a grant from the Agricultural Research Council.

REFERENCES

Dalton, A. H., Kahler, H., Kelly, M. G., Lloyd, B. J. & Striebich, M. J. (1949). *J. Nat. Cancer Inst.* **9**, 439.
Discombe, G. (1950). *Acta Haematologica*, **3**, 151.
Fishman, W. H. (1939). *J. biol. Chem.* **127**, 367.
Fishman, W. H., Springer, B. & Brunetti, R. (1948). *J. biol. Chem.* **173**, 449.
Glassman, H. N. (1950). *Science*, **111**, 688.
Graham, A. F. (1946). *Biochem. J.* **40**, 603.
Hockenhull, D. (1948). *Nature, Lond.*, **162**, 850.
Kerr, L. M. H., Campbell, J. G. & Levvy, G. A. (1949). *Biochem. J.* **44**, 487.
Kerr, L. M. H. & Levvy, G. A. (1951). *Biochem. J.* **48**, 209.
Levvy, G. A., Kerr, L. M. H. & Campbell, J. G. (1948). *Biochem. J.* **42**, 462.
Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
Opie, E. L. (1949). *J. exp. Med.* **87**, 440.
Putnam, F. W. (1948). *Advanc. Prot. Chem.* **4**, 79.
Rabinovitz, M. & Boyer, P. D. (1950). *J. biol. Chem.* **183**, 111.
Schneider, W. C. & Hogeboom, G. H. (1950). *J. biol. Chem.* **183**, 123.
Zollinger, H. (1948). *Amer. J. Path.* **24**, 569.

Degradative Studies on the 'Core' Resulting from Chymotryptic Digestion of Insulin

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Previous work on the action of enzymes on insulin (Butler, Dodds, Phillips & Stephen, 1948) has shown that chymotryptic digestion releases a number of peptide fragments which are soluble in 0·25 N-trichloroacetic acid, leaving a 'core', with a molecular weight of approximately 5000, which is precipitated. Some properties of this core have already been described (Butler, Phillips, Stephen & Creeth, 1950).

The core can be oxidized by the method used by Sanger (1949*a*) for insulin, but application of Sanger's fractionation procedure did not separate the glycyl and phenylalanyl chains as in the original insulin. Difference in solubility at pH 6·5, which in oxidized insulin distinguishes the chains, does not occur among the oxidation products of the core, presumably because of the decreased length of the chains and the removal from the phenylalanyl chains of a large proportion of the basic amino-acids by enzymic action.

The oxidation products of the dinitrophenyl derivative of the core or the dinitrophenyl or benzoyl

derivatives of the oxidized core can, however, be fractionated because of the difference in their solubility, which seems to depend on the relative content of sulphur (present as cysteic acid residues). With the exception of one, the dinitrophenyl

METHODS

Preparation of derivatives and fractionation procedures

The fractionations described below are summarized in Fig. 1.

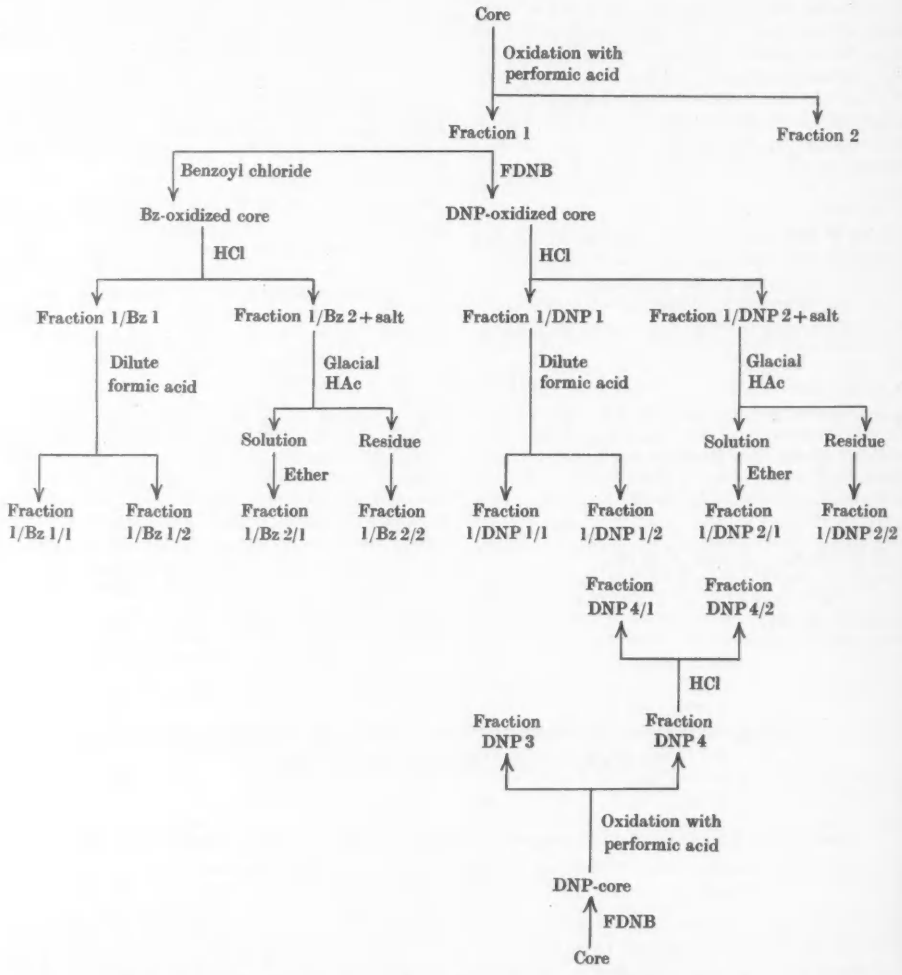

Fig. 1. Fractionation of the insulin core after oxidation and treatments with fluorodinitrobenzene (FDNB) and benzoyl chloride. For details see text.

fractions were still complex, as indicated by the multiplicity of their end groups. The one which appears to be simple has been more thoroughly investigated.

Oxidation of the dinitrophenyl-(DNP-) core. DNP-core (0·5 g.) (Butler *et al.* 1950), was treated with 18 ml. of 90% (v/v) aqueous formic acid and 2 ml. of H_2O_2 (100 vol.). The solid was broken up, and after standing for 1 hr. it had all

dissolved. Water (40 ml.) was added, which precipitated a bright-yellow solid. This was centrifuged down, washed with water and dried (wt. 160 mg., fraction DNP3). The supernatant liquid was combined with the washings and concentrated *in vacuo* in a water bath below 50°. Finally the concentrated solution was taken to dryness in a desiccator (wt. 313 mg., fraction DNP4).

Further fractionation of fraction DNP4. Fraction DNP4 (78 mg.) was dissolved in 5 ml. of water and 3 ml. of 0·1 N-NaOH (resulting pH approx. 9·5). A small amount of insoluble material was filtered off and rejected. 2·3 N-HCl (0·5 ml.) was added, which brought the pH to 1, and then a drop of 6 N-HCl. On standing for a few minutes a precipitate formed, was centrifuged down and dried (wt. 15 mg., fraction DNP4/1). The supernatant liquid was discarded as it contained such a large amount of salt.

Fractionation of the oxidized core. Oxidized core (350 mg.) (Butler *et al.* 1950) was dissolved in 10 ml. of 0·1 N-NaOH to give final pH approx. 8. A clear solution was produced. The pH was adjusted to 4·5 by the addition of 0·1 N-HCl (5 ml.) and the resulting flocculent precipitate was centrifuged down, washed with water and dried (wt. 11 mg., fraction 2). The supernatant was neutralized and evaporated to dryness in a desiccator (wt. of dry residue 358 mg., fraction 1). This contained salt. Analyses showed N (Kjeldahl), 9·7 (Dumas, by Weiler and Strauss, Oxford), 10·9 %. Assuming the N content of the core to be about 15 %, that of the oxidized core (containing eight —SO_3H groups/5000 mol.wt.) should be about 14 %. This suggests that fraction 1 contains about 260 mg. peptide material.

DNP derivative of fraction 1. Fraction 1 (323 mg.), still containing about 80 mg. NaCl, was dissolved in 10 ml. of water. 250 mg. $NaHCO_3$ were added, followed by a solution of 0·5 ml. of 1-fluoro-2:4-dinitrobenzene (FDNB) in 10 ml. ethanol. The mixture became bright yellow on shaking. It was left to stand overnight, and then evaporated to dryness. The orange residue was extracted three times with ether to remove excess FDNB, dissolved in warm water and acidified with a few drops of 6 N-HCl. A bright-yellow precipitate immediately formed, which was centrifuged down, washed successively with water, ethanol and ether and dried (wt. 210 mg., fraction 1/DNP1). It was obvious that this did not represent the total reaction product, so the yellow supernatant liquid and the washings were neutralized and evaporated to dryness in a desiccator (wt. of dry residue 328 mg., fraction 1/DNP2). This was found to contain only 4 % of N, which indicates a salt content of 70 %, assuming the true N content of the peptide to be about 14 %. The total weight of DNP products recovered, therefore, was 210 mg. insoluble material and about 95 mg. soluble.

A preparation of fraction 1/DNP2 containing about 315 mg. peptide was treated with glacial acetic acid. Ether was added to the yellow solution and a yellow precipitate was produced (wt. when dry 143 mg., fraction 1/DNP2/1). The residue which was insoluble in glacial acetic acid contained 80 mg. of peptide (fraction 1/DNP2/2). This amounted to about 13 % of the total DNP products obtained from this preparation.

Further fractionation of fraction 1/*DNP*1. Fraction 1/DNP1 (77 mg.) was dissolved in 3 ml. 90 % (v/v) formic acid and water (10 ml.) was added. An immediate precipitate was produced. This was centrifuged down, washed with water and dried (wt. 28 mg., fraction 1/DNP1/1). The supernatant liquid was evaporated to dryness (wt. of dry residue 34 mg., fraction 1/DNP1/2).

Benzoylation of fraction 1. Fraction 1 (400 mg.) was dissolved in water (20 ml.) with 1 g. of $NaHCO_3$. 0·5 ml. benzoyl chloride was added in 0·1 ml. portions with vigorous shaking. The pH was maintained at 8·3. The solution frothed when shaken and a whitish solid formed in it. The mixture was left at room temperature for 2 hr. with frequent shaking. It was then acidified with 17·5 ml. of 0·5 N-HCl to pH 2. A white solid was produced which was centrifuged down. It was extracted three times with ether to remove benzoic acid, etc. (wt. of solid 153 mg., fraction 1/Bz1). The supernatant liquid was neutralized to pH 7, and extracted with ether; after evaporation of the small amount of dissolved ether the remaining solution was freeze-dried (wt. 1·06 g., N 2·2 %, fraction 1/Bz2). From this it was estimated that the solid contained approximately 180 mg. peptide, assuming a N content 13 %, which allows for the addition of 10 benzoyl groups/mol.wt. 5000. Total benzoylated material recovered, therefore, was 333 mg., of which 46 % was insoluble in dilute HCl and 54 % soluble.

Fraction 1/Bz2 (1·06 g.) was treated with about 15 ml. of glacial acetic acid which had been dried over $CaSO_4$. The residue was filtered off and treated twice more with acetic acid. An equal volume of ether was added to the acetic acid solution, giving a white precipitate which was centrifuged down, washed with ether twice and dried (wt. 111 mg., fraction 1/Bz2/1). The insoluble residue weighed 842 mg., and contained 0·75 % N, i.e. approximately 45 mg. peptide material (fraction 1/Bz2/2).

Further fractionation of fraction 1/*Bz*1. Fraction 1/Bz1 (144 mg.) was dissolved easily in 9 ml. of 90 % (v/v) formic acid. Water (25 ml.) was added. A white precipitate was immediately formed, which was centrifuged down, washed with water and dried (wt. 65 mg., fraction 1/Bz1/1). The supernatant liquid and washings were concentrated in a water bath at about 50° *in vacuo*, and finally taken to dryness in a desiccator (wt. 60 mg., fraction 1/Bz1/2).

End-group determinations

DNP derivatives of the fractions were hydrolysed by refluxing for 8 or 12 hr. with 6 N-HCl in an oil bath at 130–140°, and the end groups were determined by the method of Mills (1950). The acid solution was extracted with approximately equal volumes of ether four times, and the ether extracts were washed once with 6 N-HCl to remove traces of free amino-acids which might have been carried over, then evaporated to dryness in a small test tube. A few drops of a saturated solution of $Ba(OH)_2$ were added to the residue and the tube was placed in a boiling-water bath for 0·5–1 hr. Excess Ba was precipitated as $BaCO_3$ by the addition of a small piece of solid CO_2 and centrifuged off. The supernatant liquid was drawn off in a micropipette, evaporated to dryness and the residue applied to a two-dimensional paper chromatogram, which was developed by the method of Consden, Gordon & Martin (1944).

Where the presence of *N*-terminal glycine was suspected, this had to be determined separately, because DNP-glycine is not stable to prolonged hydrolysis, nor is glycine efficiently regenerated by hydrolysis of DNP-glycine with $Ba(OH)_2$. Consequently, the hydrolysis procedure of Sanger (1949*b*) was used. A sample of the acid hydrolysate was withdrawn after 2 hr. and extracted with ether. The ether extract after evaporation was run on a series of buffered silica columns. First, it was run on silica moistened with 0·25 M-sodium phosphate buffer (pH 8), using a water-saturated solution

(10%, v/v) of *n*-butanol in $CHCl_3$ as moving phase. Secondly, it was run on silica buffered with 0·5M phosphate (pH 8), using a water-saturated solution (33%, v/v) of ether in ethyl methyl ketone. Any likely bands were compared with those given by authentic DNP-glycine on similar columns; the material from them was then mixed with DNP-glycine and, if no separation occurred on one of these columns, the unknown was taken to be DNP-glycine.

Enzymic digestions of fractions

Crystalline pig pepsin was obtained from Parke Davis and Co. Ltd., papain from May and Baker Ltd. and pancreatin from British Drug Houses Ltd. Papain and pancreatin were used without further purification, but the papain was activated with HCN according to the method of Anson (1938).

Solutions of the DNP fractions (approximately 5 mg./ml.) were prepared by dissolving in water with the addition of drops of 0·1N-NaOH to pH 8–8·5 for pancreatin, or pH 5 for papain, or with the addition of 0·1N-HCl to pH 2 for pepsin digests. The enzymes were used in concentrations of approximately 5×10^{-4} unit/ml. digest at 25° for at least 24 hr., and in some cases 48 hr.

RESULTS

DNP fractions from the core and oxidized core

In five preparations from two different preparations of core, the amount of insoluble product, fraction DNP3, expressed as percentage by weight of the original DNP-core, maintained a fairly uniform proportion, as shown in Table 1.

Table 1. *Yield of insoluble fraction from oxidation of DNP-core*

Preparation	Core	Fraction DNP3 (%)
1	C16B	43
2	C16B	32
3	C16B	29
4	C16B	40
5	C15B	29

Benzoylated fractions from the oxidized core

Yields of the fractions, which were almost the same in three different preparations are shown in Table 2.

Table 2. *Yields of benzoylated fractions from oxidized core*

(Percentages by wt. of aggregate wt. of fractions.)

	Insoluble			Soluble		
Benzoylation	Bz1→Bz1/1 + Bz1/2			Bz2→Bz2/1 + Bz2/2		
1	43	26	17	57	—	—
2	46	24	22	54	39	15
3	45	27·5	17·5	55	42	13

Benzoylation of the core followed by oxidation was not successful.

The four benzoyl fractions, Bz1/1, Bz1/2, Bz2/1, Bz2/2, are assumed to correspond to the four DNP fractions, DNP1/1, DNP1/2, DNP2/1, DNP2/2.

Analysis of fractions

DNP fractions 1/DNP1,1/DNP2/1,1/DNP1/1,1/DNP 1/2, DNP 3 and DNP 4 and benzoyl fractions 1/Bz2/1, 1/Bz1/1, 1/Bz1/2 were analysed for S and N by Drs Weiler & Strauss. Results corrected for ash and moisture content are given in Table 3, where corresponding DNP and benzoyl fractions are shown side by side.

From these results it can be seen that the solubility which in the case of the DNP fractions follows the order

$$\frac{1/\text{DNP}2/1}{\text{DNP}4} > 1/\text{DNP}1/2 > \frac{1/\text{DNP}1/1}{\text{DNP}3}$$

and in the case of benzoyl derivatives

$$1/\text{Bz}2/1 > 1/\text{Bz}1/2 > 1/\text{Bz}1/1$$

seems to depend on the amount of S present in the fraction.

Cysteic acid in two benzoyl fractions, 1/Bz2/1, 1/Bz2/2, was determined by the method of Naftalin (1948) on paper chromatograms run in butanol-acetic acid mixture. 1/Bz2/1 contained 6·0% S and 1/Bz2/2, 5·9% by this method.

Terminal amino-acids

The *N*-terminal amino-acids of the cores from which these fractions were derived were glycine, valine and leucine. The distribution of these end groups amongst the DNP fractions can be seen in Table 4.

It will be seen that only in the case of fraction 1/DNP2/2 is the fraction completely homogeneous as shown by its end

Table 3. *Analysis of DNP and benzoyl fractions of oxidized core*

DNP fraction	S (%)	N (%)	Benzoyl fraction	S (%)	N (%)
1/DNP1	2·47	13·9	—	—	—
	1·93	15·1	—	—	—
1/DNP2/1	3·87	14·5	1/Bz2/1	5·22	11·65
				5·30	12·17
				5·02	10·20
1/DNP1/1	1·65	14·3	1/Bz1/1	1·46	12·98
				1·41	12·49
				2·48	12·56
1/DNP1/2	2·52	15·2	1/Bz1/2	2·77	10·39
				3·49	10·64
DNP3	1·51	17·1	—	—	—
DNP4	4·03	14·2	—	—	—
	3·66	13·3	—	—	—

group. Other fractions were apparently mixtures of chains in which one terminal amino-acid might predominate over the others, as for instance glycine does in the soluble fractions.

Chromatography on starch and alumina columns and on paper caused some further separation, but did not lead to products with single end groups.

Table 4. *Terminal amino-acids of DNP fractions*

(+, present; ++, very marked; −, weak; o, absent.)

Fraction	Glycine	Valine	Leucine
1/DNP1	+	++	+
1/DNP2	+	+	−
DNP3	+	++	−
DNP4	++	−	?
1/DNP2/1	+	+	+
1/DNP2/2	++	o	o

Distribution of amino-acids in fractions

Benzoyl fractions. These fractions were examined by paper chromatography after hydrolysis with 6 N-HCl. Results are shown in Table 5.

Table 5. *Qualitative amino-acid composition of the benzoyl fractions*

(+, present; o, absent; −, faint; ?, doubtful.)

	Fractions			
Amino-acid	1/Bz1/1	1/Bz1/2	1/Bz2/1	1/Bz2/2
Cysteic acid	+	+	+	++
Aspartic acid	+	+	+	−
Glutamic acid	+	+	+	+
Serine	+	+	+	+
Glycine	+	+	+	+
Alanine	+	+	+	+
Valine	+	+	+	+
Leucine isomers	+	+	+	+
Tyrosine	+	+	+	+
Phenylalanine	?	?	?	o
Threonine	o	o	o	o
Histidine	+	+	+	o
Arginine	?	o	?	o
Lysine	?	o	?	o
Proline	o	o	o	o

According to these results, threonine and proline are absent from all fractions. The presence of such amino-acids as phenylalanine, arginine and lysine was doubtful. The main conclusion to be reached was that the fraction 1/Bz2/2 contained neither basic amino-acids, threonine nor phenylalanine.

DNP fractions. Of the DNP fractions examined, 1/DNP2/2 was the only simple fraction, containing only one end group, DNP-glycine. A two-dimensional chromatogram of the acid hydrolysate showed the presence of the following amino-acids: cysteic, aspartic and glutamic acids, serine, alanine, valine and leucine isomers. Glycine, phenylalanine, threonine and basic amino-acids were absent. Tyrosine would be present as *O*-DNP-tyrosine, which is not easily distinguished on paper chromatograms.

The molecular weight of fraction 1/DNP 2/2

The average molecular weight of this peptide fraction 1/DNP2/2 was determined by Mr G. L. Mills by a method to be published later, involving the reduction of the nitro groups in DNP-amino-acids by titanous chloride. He estimated the amount of DNP-glycine present in an acid hydrolysate after its separation by paper chromatography. Allowing for 60% breakdown of the DNP-glycine during hydrolysis for 8 hr. in 6 N-HCl (a correction given by Sanger, 1945), he found that 1 g. residue of glycine would be present in 2000 g. There should be two DNP groups present, one terminal and one on a tyrosine residue; in this case therefore the mol.wt. of the unsubstituted peptide is approximately 1700.

This is more than was at first thought, when it was considered that the core was composed of four chains having mol.wt. about 1200 each. The figure is confirmed by other determinations. For example, cysteic acid determined by Naftalin's method gave 2 g. residues/2000 g. for 1/DNP2/2, and 3 g. residues/1600 g. for 1/Bz2/2, the corresponding benzoyl-fraction. Therefore the mol.wt. of this fraction 1/DNP2/2 can be taken as 1700–2000.

The action of enzymes on DNP fractions

The treatment of some DNP fractions with enzymes such as pepsin, papain and pancreatin showed that DNP-peptides can be split off and extracted from the acidified digest into ether. The ether-soluble material is probably not a single peptide, particularly as these fractions are known to be complex, containing more than one end group. However, the fact that they are soluble in ether shows that they must contain a DNP group, derived either from histidine, tyrosine

Table 6. *Composition of peptides extracted by ether from DNP fractions digested with enzymes*

	Composition of peptides split off by		
Fraction	Papain	Pepsin	Pancreatin
1/DNP1	Glutamic acid Serine or glycine Alanine Leucine and/or isoleucine Valine	Glutamic acid Serine, Glycine (?) Alanine Leucine and/or isoleucine Valine Aspartic acid (?)	Glutamic acid Glycine Alanine Leucine and/or isoleucine Valine Aspartic acid
1/DNP2/1	—	Aspartic acid, Alanine, Leucine: Very faint	—
1/DNP2/2	None	None	Glutamic acid, leucine, isoleucine, valine, DNP-glycine

or the terminal amino-acid. The amino-acid composition of these mixed peptides was determined by paper chromatography after acid hydrolysis, and is shown in Table 6.

The peptide obtained by peptic digestion of fraction 1/DNP1 contained *N*-terminal glycine. This was of interest because 1/DNP2/2, which originally contained terminal glycine, was unaffected by pepsin, and 1/DNP2/1 only very slightly so. It suggests, therefore, that the glycyl chains of the core may be of two different kinds, one present in fraction 1/DNP1 and the other in fractions 1/DNP2/1 and 1/DNP2/2. This means that one type must be derived from the phenylalanyl chains of the original insulin as there is only one glycine residue present in the glycyl chains thereof (Sanger, 1949*a*).

Pancreatin digested both 1/DNP1 and 1/DNP2/2. The peptide from 1/DNP2/2 contained the amino-acids valine, glutamic acid, leucine and isoleucine, also DNP-glycine; the leucine isomers were separated on a paper chromatogram in 2-dimethylpropan-1-ol in the presence of diethylamine (Work, 1949). The sequence of these amino-acids was not determined, but it appears likely that they are derived from a peptide similar to that isolated by Sanger (1949*b*) from the glycyl chains of insulin, DNP-glycylisoleucylvalylglutamylglutamic acid.

DISCUSSION

The core of the insulin molecule left after digestion with chymotrypsin contains 60–70 % of the original cystine (Butler *et al.* 1950). It is regarded as having the same form as the original insulin molecule, consisting of four peptide chains of relatively shorter lengths, held together by three or four disulphide bridges.

Fig. 2 shows the possible structures of the core derived from two of the structures of insulin proposed by Sanger (1948). The closed system for the core has been disregarded because it is thought that after oxidation it would not lead to the fractionation which occurs, because of the equal number of cysteic acid residues which each chain would contain.

Rupture of these disulphide linkages by oxidation with performic acid no longer permits ready separation of two dissimilar types of chain as in oxidized insulin. This is possibly due to the removal of most of the arginine and lysine from the phenylalanyl chains and to their decreased length, which would render them more soluble. The glycyl and phenylalanyl chains of oxidized insulin have four and two cysteic acid residues respectively. In the oxidized core the chains should now contain one, two or three cysteic acid residues.

Conversion of the disulphide linkages to sulphonic acid residues by oxidation markedly influences the solubility of the chains, which is further increased by the masking of the basic groups with groups such as dinitrophenyl or benzoyl. The mixture can then be separated easily into four main fractions.

This distinction is not clear-cut, as all DNP fractions except one contain more than one end group, signifying more than one type of chain. This may mean that chains with less sulphur are digested down to a shorter length, thereby increasing their solubility to that of longer chains with more sulphur. There are, of course, other factors which undoubtedly influence the solubility as well, which are as yet undetermined. Moreover, sulphur analyses of the fractions isolated according to this difference in solubility show that the more soluble the fraction, the higher is its sulphur content.

Further separation of these fractions by chromatography did not resolve the peptides present, although in some cases it diminished the predominance of one component in favour of another. Nor did chromatography on paper, starch or alumina produce any fraction with a single end group.

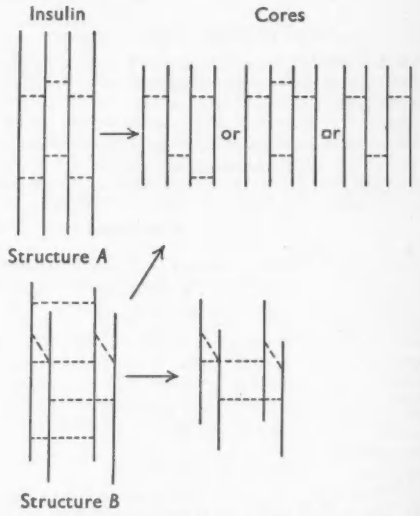

Fig. 2. Possible structures of insulin and the cores. Vertical lines represent the peptide chains and the dotted horizontal lines the disulphide bridges.

One fraction, however, which amounts to about 12 % of the whole core, has been obtained both in benzoyl and dinitrophenyl preparations of the oxidized core, and appears to have a single *N*-terminal amino-acid, glycine. The fraction contains no basic amino-acids, and a relatively high proportion of sulphur. It has a molecular weight of 1700–2000.

The glycyl chains of insulin have a molecular weight of 2750 (Sanger, 1949*b*), so that only about seven amino-acid residues have been split from the intact chain to form the new glycyl fraction. If all chains of the core were of approximately the same length, their molecular weight would be about 1200. Presumably some chains with less sulphur are shorter to compensate for this long one. The disulphide bridges in the original molecule probably provide a barrier to the action of the enzyme so that

the chains with a greater number of these are not digested down so far as those with fewer.

The action of pancreatin on this peptide has revealed that it terminates in a peptide containing DNP-glycine, isoleucine, valine and glutamic acid, which Sanger has described as a *N*-terminal peptide sequence from the glycyl chains of insulin. It also contains an additional leucine residue. The fraction is therefore probably derived from the *N*-terminal end of the original glycyl chains and digestion by the enzyme apparently occurs from the other end of the chain.

This fraction 1/DNP2/2 is unaffected by other enzymes such as pepsin and papain; this distinguishes it from the glycyl chains in another complex fraction 1/DNP1, which are digested with pepsin. This suggests that not all the glycyl chains in the core are alike. Unless rearrangements are brought about by the enzymes the latter glycyl chains must therefore come from the phenylalanyl chains of insulin. The chains having valyl or leucyl end groups may be derived from either type of original chain.

SUMMARY

1. The dinitrophenyl derivative of the 'core' resulting from chymotryptic digestion of insulin has been oxidized with performic acid, and three fractions, which differ in solubility, have been isolated.

2. The core itself has been oxidized, and the main product after treatment with fluorodinitrobenzene or benzoyl chloride has in each case been separated into four fractions.

3. These benzoyl or dinitrophenyl fractions have been analysed for sulphur.

4. The dinitrophenyl fractions, with the exception of one, are complex, having a variety of *N*-terminal amino-acid residues.

5. One dinitrophenyl fraction, about 13% of the total product, has a glycyl end group, a molecular weight of about 2000, and a high cysteic acid content. A terminal peptide, yielding on acid hydrolysis *N*-dinitrophenylglycine, isoleucine, valine, glutamic acid and leucine, has been split from it by pancreatin.

I acknowledge with pleasure the interest of Prof. E. C. Dodds, F.R.S., in this work, and helpful advice from Dr J. A. V. Butler. I am also grateful for discussions with Dr D. M. P. Phillips, and for the molecular-weight determinations carried out by Mr G. L. Mills. Work reported in this paper formed part of a thesis submitted to the University of London for the Ph.D. degree.

REFERENCES

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Butler, J. A. V., Dodds, E. C., Phillips, D. M. P. & Stephen, J. M. L. (1948). *Biochem. J.* **42**, 116.
Butler, J. A. V., Phillips, D. M. P., Stephen, J. M. L. & Creeth, J. M. (1950). *Biochem. J.* **46**, 74.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Mills, G. L. (1950). *Nature, Lond.*, **165**, 403.
Naftalin, L. (1948). *Nature, Lond.*, **161**, 763.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1948). *Nature, Lond.*, **162**, 491.
Sanger, F. (1949*a*). *Biochem. J.* **44**, 126.
Sanger, F. (1949*b*). *Biochem. J.* **45**, 563.
Work, E. (1949). *Biochim. Biophys. Acta*, **3**, 400.

The Spectrophotometric Determination of Ascorbic Acid in Tissue Extracts, Particularly those of the Walnut (*Juglans regia*)

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(*Received* 27 *January* 1951)

Although ascorbic acid shows a well defined absorption band in the ultraviolet region of the spectrum, this property has received little attention for the determination of the vitamin in tissue extracts. The reasons for this would seem to be that the band is in a region where many other substances interfere, that the position of the band varies with pH (Karrer, Salomon, Morf & Schöpp, 1933) and that beyond certain limits the intensity of absorption is not directly proportional to the concentration (Herbert, Hirst, Percival, Reynolds & Smith, 1933).

Previously described spectrophotometric methods of assay have depended upon the measurement of absorption, before and after the destruction of ascorbic acid, by oxidation in the presence of copper (Johnson, 1936) or by irradiation (Chevallier & Choran, 1937). These methods are not applicable to extracts which contain a second reducing agent which absorbs in the same region as the ascorbic acid. Loureiro (1936) suggested that the shift with pH of the absorption curve might be utilized for determining ascorbic acid, but gave few details.

The factors affecting the position and intensity of absorption have been studied, and a spectrophotometric method of assay developed, details of which are described below.

EXPERIMENTAL

General. Absorption measurements were made with a Beckman spectrophotometer, using 1 cm. quartz cells and a hydrogen discharge lamp. Solutions were prepared with water freshly distilled from glass. The buffer solutions used were those described by Daglish, Baxter & Wokes (1948), and acetate buffers to cover the range pH 3·8–5·7. pH measurements were made with a Cambridge pH meter.

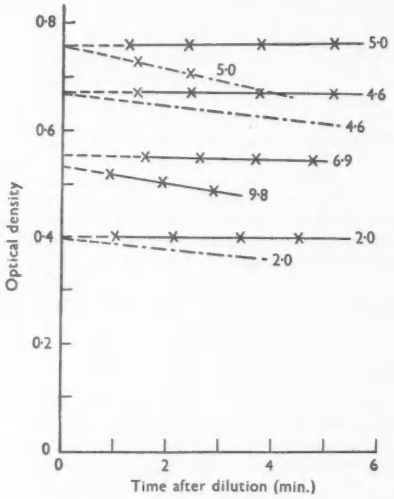

Fig. 1. The effect of instrumental irradiation on the stability of ascorbic acid solutions at different pH values. ——, with KCN; —·—·, without KCN. Figures denote pH values.

Stability of ascorbic acid during assay. A stock solution (1 mg./ml.) of ascorbic acid in 0·01 N-HCl was diluted to 6–8 μg./ml. in various buffers, with and without the addition of KCN solution (1 mg./ml.), to give a final concentration of 10 μg./ml. The optical density at the absorption maximum was read at 60 sec. intervals. The results (Fig. 1) showed that irradiation of ascorbic acid solutions during measurement caused considerable losses. Traces of heavy metals may have been responsible in part for these losses which were prevented by KCN at pH values less than 6, but above this it was less effective.

Effect of pH *on the absorption curve of ascorbic acid*

The absorption curve was determined from 220–300 mμ. for dilutions of ascorbic acid and KCN in buffers covering the range pH 0–11. With unstable solutions, the density at the maximum was determined at timed intervals, and the value at zero time obtained by extrapolation.

The results, when plotted (Fig. 2), gave a family of absorption curves passing through an isosbestic point at 250 mμ., where $E^{1\%}_{1\,cm.}$ is 485·3. This is due to the transition from the non-ionized form of the molecule absorbing at 245 mμ., to the totally ionized

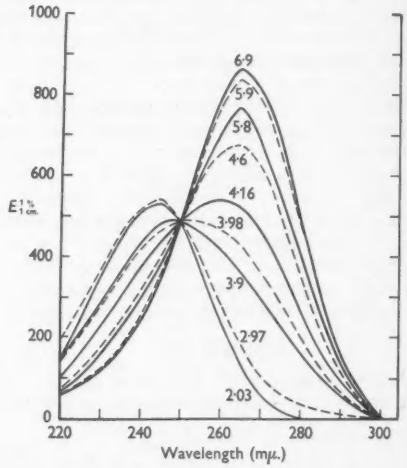

Fig. 2. The effect of pH upon the absorption curve of ascorbic acid. (Figures adjoining curves denote pH.)

anion absorbing at 265 mμ. The pK_1 value of ascorbic acid calculated from the data is 4·16, which agrees with 4·17 given by Rosenberg (1942), where

$$pK_1 = pH + \log \frac{[\text{Unionized form}]}{[\text{Ionized form}]},$$

and from which the dissociation constant is $6{\cdot}92 \times 10^{-5}$.

PROPOSED METHOD OF ASSAY

Fig. 2 shows that if the absorption of ascorbic acid is measured first at a pH below 3·5 and then above 4·5, two distinct curves will be obtained with peaks at 245 and 265 mμ. respectively as in Fig. 3*a*. The differences in readings of the two curves at 245 and 265 mμ., x and y, will depend only on the concentration and the selected pH values. Hence, if these last are kept constant, x and y will be proportional to concentration and the ratio y/x will be a constant for ascorbic acid.

The assay of ascorbic acid in any extract consists then of diluting into two buffers and measuring the values of x and y. If these are known for the pure compound, its proportion in the extract can be readily calculated. When the ratio y/x for the extract

is the same as that for ascorbic acid, the method is specific for this compound, and any discrepancy will indicate the presence of interfering substances.

The choice of buffers will depend largely upon the stability of the other constituents of the extract, and upon any shift in absorption they might exhibit. Two buffers giving an y/x ratio of the extract approximating that for ascorbic acid should be used.

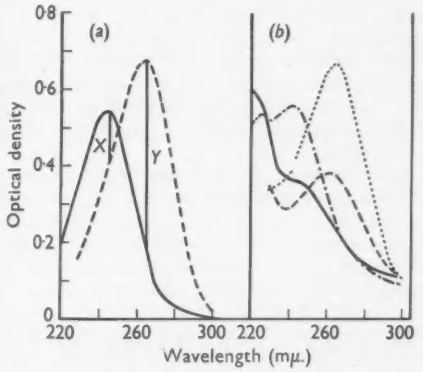

Fig. 3. *a*, absorption curve of ascorbic acid at (——) pH 3 and (— — —) pH 4·6. *b*, absorption curve of mesocarp extract at (——) pH 3 and (— — —) pH 4·6; and of endocarp extract at (—·—·—) pH 3 and (. . . . . .) pH 4·6.

Application of assay to walnut extracts

Walnut extracts are unstable above pH 5 (Daglish, 1950*a*) so buffers of pH 3·0 and 4·6 were chosen for the assay. Dilutions in these of two extracts gave the curves of Fig. 3*b*, whilst ascorbic acid gave those of Fig. 3*a*. The fact that the y/x ratio determined for a number of extracts did not differ significantly from the value 3·62 found for ascorbic acid (Table 1) confirmed that these buffers were satisfactory. A change of pH in this range gave negligible shift to the curves of isolated hydrojuglone glucoside, tannins and their accompanying colouring matters.

Ethanolic hydrochloric acid had proved the most satisfactory solvent for stabilizing hydrojuglone glucoside (Daglish, 1950*a*). In this, or in ethanol, ascorbic acid solutions are unstable at room temperature, but more stable when refrigerated (Table 2). To avoid losses before assay, the following procedure was adopted.

Freshly prepared extract (2 ml.), diluted with 0·01 N-hydrochloric acid to a final volume of 10 ml., was chilled to precipitate and coagulate chlorophyll and other water-insoluble constituents. The clear solution, clarified by spinning if necessary, was diluted into the buffers with sufficient potassium cyanide solution (1 mg./ml.) to give a final concentration of 10 μg./ml. of this compound, and the absorption measured at 245 and 265 mμ., then at 5 mμ. intervals to cover the range 230–280 mμ. The absorption curves of dilutions of a freshly prepared ascorbic acid solution (1 mg./ml. in 0·01 N-hydrochloric acid) were determined for each group of assays to avoid variations due to slight changes in the buffer solutions.

This spectrophotometric method of assay and the formaldehyde dye titration, previously described (Daglish, 1950*b*) were applied to extracts prepared from different tissues of the walnut throughout 1949 and 1950. This was to determine whether a similarity

Table 1. *Comparison of spectrophotometric assay results with those of formaldehyde dye titration*

	Ascorbic acid	Walnut extracts			
		Mesophyll	Epicarp	Mesocarp	Endocarp
No. of assays	18	18	15	15	15
$\frac{\text{Spectrophotometric assay} \times 100}{\text{Dye titration assay}}$	—	93·5	101·7	87·5	103·5
Deviation of mean	—	6·5	3·1	4·7	1·2
y/x	3·62	3·48	3·70	3·57	3·33
Deviation of mean	0·16	0·55	0·29	0·24	0·21

Table 2. *Stability of* 0·07 % *(w/v) solution of ascorbic acid in ethanol and ethanolic hydrochloric acid*

(The results are expressed as a percentage of the amount originally present.)

HCl (N)	Temp. (°)	Percentage after				
		3 hr.	22 hr.	46 hr.	70 hr.	120 hr.
0	18	88	59	21·7	—	—
0	4	100	100	100	98·2	83·6
0·3	18	70·6	0	—	—	—
1·5	18	98·8	71	25	—	—
1·5	4	100	97·6	87·4	69·7	40·0
3·0	18	101·8	90·9	46·3	—	—
Catkin extract 0·3 N	4	—	96·3	96·3	85·3	—

in results was obtained for the distribution of ascorbic acid in the plant, which could be compared with the results of earlier workers who had used only the dye titration. Although a detailed account of this work will be given in a future paper, the results of sixty-three such duplicate assays are summarized in Table 1. Fig. 4 shows the seasonal variation in concentration of ascorbic acid in two tissues when determined by both methods.

Dehydroascorbic acid. Because this does not absorb at 245 or at 265 mμ., it was determined by the difference in the reduced ascorbic acid values found after and before treatment with hydrogen sulphide at pH 3.

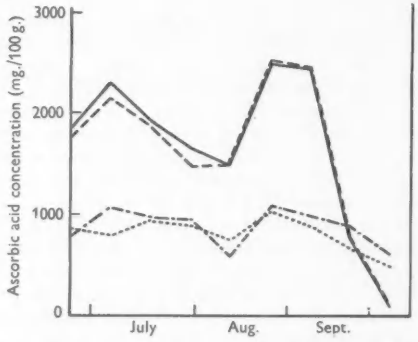

Fig. 4. Seasonal variation of ascorbic acid concentration in endocarp and mesocarp. ——, endocarp by spectrophotometric assay; — — —, endocarp by indophenol dye titration; . . . . . , mesocarp by spectrophotometric assay; —·—·—, mesocarp by indophenol dye titration.

Accuracy of the method. The accuracy depends upon the least density difference at 245 and 265 mμ., caused by the change in pH, which can be measured, and upon the x and y values of ascorbic acid in the selected buffers. At pH 3·0 and 4·6, the changes in $E^{1\%}_{1\,cm.}$ at 245 and 265 mμ. are $-131{\cdot}4$ and $+474$ respectively. If the reproducible least density difference is taken as 0·005 (which is equivalent to 0·038 and 0·011 mg./100 ml. ascorbic acid at the two wavelengths), and if the concentration for assay is about 0·8 mg./100 ml., the error is 1·3 and 4·7 % at 265 and 245 mμ. respectively.

DISCUSSION

Owing to the complex nature of most natural products, it is desirable to minimize the number of steps in any assay method to prevent the formation of non-specific substances and the destruction of ascorbic acid. To overcome these difficulties, a general procedure has arisen of determining the total effect (indophenol titration, ultraviolet light absorption, etc.) before and after the destruction of ascorbic acid. Should a second substance be present in the extract as, for example, hydrojuglone glucoside in the walnut, which is affected by the oxidation step, the results are often doubtful.

Loureiro (1936) suggested that the shift with pH of the ultraviolet absorption might be applied to the determination of ascorbic acid. Although the shift was characteristic of a group of enediols, the positions of the maxima of those investigated were sufficiently far removed from that of ascorbic acid to avoid interference. Reductone, for example, would not interfere unless present to the extent of 30 % the amount of ascorbic acid. He proposed measurement at pH 1 and 6–7, but gave no values for the extinction of ascorbic acid, save that it was lower at the shorter wavelengths.

After investigating the effect of pH on the absorption curve of ascorbic acid it has been found possible to develop a method of assay applicable to walnut extracts. Measurements were made at pH 3·0 and 4·6. A change of pH in this range gave negligible shift to the absorption curves of other constituents of the extracts as shown by the agreement of the y/x values of these when compared with that of ascorbic acid. This agreement indicates that the method is specific for this compound, and a deviation, the presence of interfering substances. A further and more rapid check on the specificity of the method is the intersection of the curves at 250 mμ., the isosbestic point of ascorbic acid (Fig. 2).

In this assay no purification step was necessary save that of removing water-insoluble substances from acid ethanol extracts. It was found to be much quicker than the indophenol dye titration in that only 6–8 min. were required for determining the two sets of absorption readings. Comparison of the results by the two methods (Table 1) showed that good agreement was reached when ascorbic acid alone was present as in endocarp extracts, but greater variations were experienced when a second dye reductant (hydrojuglone glucoside) was present as in the mesocarp and epicarp.

Whilst the present work was in progress, a similar method of assay was proposed by Vacher & Faucquembergue (1949). The treatment of the results by these workers differs from that above, in that the 'shift curve' is plotted and identified with that of ascorbic acid, when the pH is moved from below pH 3·5 to slightly above 5·5. They suggest that accurate control of the pH is not necessary below pH 3·5 and above 5·5 provided 7·5 is not passed. Fig. 2 shows that the extinction values of the maxima in these regions vary greatly with pH, and for accurate work the pH must be closely controlled.

Although applied in the present work only to walnut extracts, the method of assay should be applicable, as found by the French workers, to the determination of ascorbic acid in most tissue extracts.

SUMMARY

1. The absorption characteristics of ascorbic acid as affected by pH have been determined.

2. A method of assay utilizing the shift of the absorption curve has been suggested.

3. The results of this method when applied to walnut extracts have been compared with those obtained by the indophenol dye titration.

I wish to thank Dr F. Wokes, the Director of the Laboratories, for affording me the opportunity to publish this work (which, together with that described in the following paper, will form part of the thesis for a Ph.D. degree of the University of London), and Miss N. Baxter for technical help in the preparation of the many extracts used.

REFERENCES

Chevallier, A. & Choran, Y. (1937). *Bull. Soc. Chim. biol., Paris*, **19**, 511.

Daglish, C. (1950*a*). *Biochem. J.* **47**, 452.

Daglish, C. (1950*b*). *Biochem. J.* **47**, 458.

Daglish, C., Baxter, N. & Wokes, F. (1948). *Quart. J. Pharm.* **21**, 344.

Herbert, R. W., Hirst, E. L., Percival, E. G. V., Reynolds, R. J. W. & Smith, F. (1933). *J. chem. Soc.* p. 1270.

Johnson, S. W. (1936). *Biochem. J.* **30**, 1430.

Karrer, P., Salomon, H., Morf, R. & Schöpp, K. (1933). *Biochem. Z.* **258**, 4.

Loureiro, M. J-A. de (1936). *Bull. Soc. Chim. biol., Paris*, **18**, 757.

Rosenberg, H. R. (1942). *Chemistry and Physiology of the Vitamins*, p. 295. New York: Interscience.

Vacher, M. & Faucquembergue, D. (1949). *Bull. Soc. Chim. biol., Paris*, **31**, 1419.

The Occurrence of Ascorbic Acid in the Walnut (*Juglans regia*)

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In an earlier paper (Daglish, 1950*a*) it was shown that hydrojuglone glucoside was more resistant to atmospheric oxidation in walnut extracts than in solutions prepared therefrom by chromatography. The properties of the antioxidant suggested that it was ascorbic acid, high concentrations of which are known to occur in the walnut (Gerghelezhiu, 1937).

Breinlich (1942) postulated that ascorbic acid and juglone formed part of an oxidation-reduction system. If there is a similar interaction between ascorbic acid and hydrojuglone glucoside, then, as the concentration of the latter shows definite seasonal variations in the different tissues of the plant (Daglish, 1950*b*), it would be expected that ascorbic acid would exhibit corresponding variations. In order to determine if this is so, the occurrence of ascorbic acid has been determined throughout the 1949 and 1950 growing periods.

EXPERIMENTAL

Preparation of extracts. In order to avoid variations due to location, age of trees, soil, etc., samples were taken from one mature tree in 1949. The ascorbic acid concentration in the different plant parts exhibited definite seasonal fluctuations. A second tree in a different location was used in 1950 to demonstrate that these variations were characteristic of the walnut as a species and not just a single member.

Within 30 min. of collection, the plant material was extracted into ethanolic HCl (Daglish, 1950*a*), and then assayed for ascorbic acid, dehydroascorbic acid and hydrojuglone glucoside by the spectrophotometric methods previously described (Daglish, 1950*b*, 1951). All results were calculated on a fresh weight basis.

RESULTS AND DISCUSSION

Male catkins. To minimize errors caused by the variation of individual catkins, at least twenty of these were used for each assay. Duplicate assays at any one time differed by not more than 8·5 %, which was less than the variation of successive weekly samples.

When first distinguishable from the vegetative buds, the male catkin buds weighed 30–50 mg. each, and contained approximately 30 mg./100 g. of ascorbic acid. This level was maintained throughout the winter. In the spring there was an initial gradual increase in weight to 0·1–0·15 g. and with this, the concentration of ascorbic acid increased to 160 and 386 mg./100 g. in 1950 and 1949, respectively. This was followed by a rapid decrease in concentration which was not due to a loss of ascorbic acid, but due solely to the 20-fold increase in weight which had taken place. In fact, the amount of ascorbic acid present showed an increase as indicated

by the average amount per catkin. This rose from less than 0·25 mg. in the winter resting state to 1·0–1·5 mg. at 50% full growth, then remained constant or fell slightly. The possible sources of this extra ascorbic acid were, at first, the cortex and phloem of the adjoining stem, and later, by translocation from the unfolded leaves or by photosynthesis in the green catkins themselves. Some slight evidence for this was obtained both from ringing experiments and by growing catkins on isolated branches in water.

Rings of bark were removed from stems of trees before any signs of development in the catkins were visible. Catkins which developed above the rings were very stunted in growth, averaging 20–25% the weight of normal catkins, were brown rather than green in colour, and showed no ascorbic acid on assay. In contrast catkins, grown on isolated branches in water, developed to maturity and formed and shed pollen. The vegetative buds on the same stems, however, remained dormant. The weight of the catkins was again only 25% that of controls and no ascorbic acid could be detected.

It would thus appear that ascorbic acid was necessary for full and rapid growth, and that in the catkins this was obtained, first of all from the adjoining stem, then later from the leaves by translocation.

Vegetative buds. As with male catkin buds, precautions were taken to minimize sampling errors and those due to individual variations in vegetative buds. These were first detected in late June when they weighed about 30 mg. each. With the increase in weight to 50–60 mg. in the same season, the concentration of ascorbic acid increased from 100 to 450 mg./100 g. As this level was approximately the same as that in the leaf stalk, it suggested that the ascorbic acid was received from the mesophyll. After reaching full size the concentration fell steadily, until in the winter dormant state it was difficult to detect any ascorbic acid.

With the development of the buds in the spring the concentration rose rapidly to 300 mg./100 g. and this rise was continued in each of the tissues derived from the buds. As with all the other tissues of the plant investigated, the increase in concentration of ascorbic acid occurred just before the increase in growth.

Mesophyll, midribs and stalks. The seasonal variations in the concentration of ascorbic acid in these tissues are shown in Fig. 1, together with the growth curve of the leaflets. The rapid growth in the early part of the summer was preceded by a similar increase in the concentration, which reached in the mesophyll 750 and 850 mg./100 g. in 1949 and 1950, respectively. The midribs and stalks showed somewhat lower levels, and except at the very beginning of growth, there was a concentration gradient from the mesophyll into the stalks which was maintained throughout the growing season. The initial reverse flow suggested that the bud residues were the source of ascorbic acid required by the unfolding leaves until they were able to synthesize this.

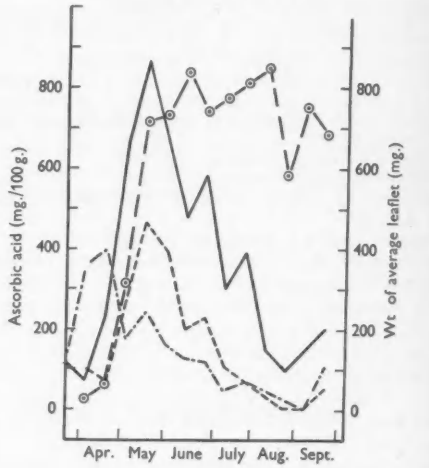

Fig. 1. The seasonal variation in concentration of ascorbic acid during 1950. ——, mesophyll; — — —, midribs; —·—·—·—, stalks; ⊙—⊙, weight (mg.) of average leaflet.

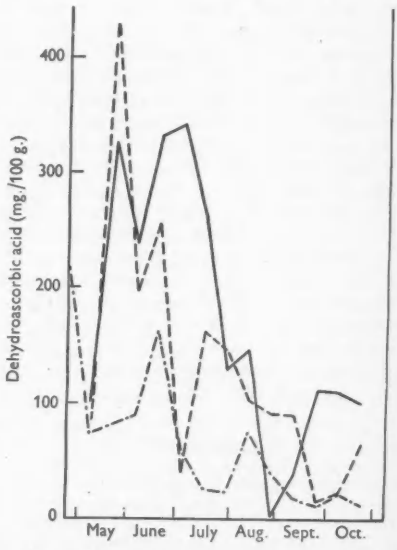

Fig. 2. Seasonal variation in concentration of dehydroascorbic acid during 1950. ——, mesophyll; — — —, midribs; —·—·—·—, stalks.

A similar gradient from the mesophyll was established for dehydroascorbic acid (Fig. 2). This showed a maximum concentration at the same time as the reduced form, after which there was a steady fall in both until the leaf fall.

Stem. The concentration of ascorbic acid in the stem was only 150–200 mg./100 g. at its maximum. These figures were obtained on the outer cortex and phloem. The xylem and pith gave still lower values. As these concentrations were lower than that in the stalks, it would appear that the stem provided a path of conduction of ascorbic acid from the photosynthetic centres of the mesophyll to the rest of the plant.

In contrast, the concentration of dehydroascorbic acid remained fairly constant at 250 mg./100 g. throughout the year, and it is possible that it forms a source of ascorbic acid for the developing plant parts in the following year.

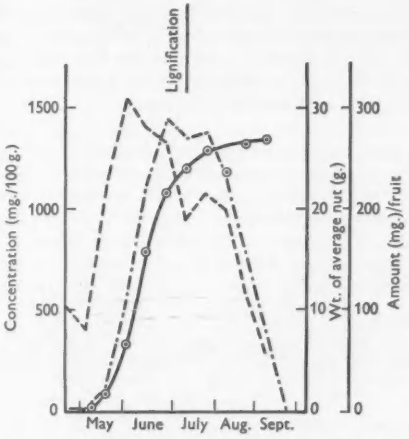

Fig. 3. The effect of growth upon the concentration (mg./100 g.) and amount (mg.) per fruit of ascorbic acid in whole fruits. ⊙—⊙, growth; — — —, concentration; —·—·—·, amount per fruit.

Fruits. Fig. 3 shows the relationship between the increase in weight and the amount and concentration of ascorbic acid in whole fruits for 1950. An almost identical figure was obtained for 1949, although the concentration was somewhat higher (2000 mg./100 g. as compared with 1500 mg./100 g. for 1950). The concentration reached its peak when the fruits weighed 1–2 g., whereas the average amount per fruit was a maximum when they were fully grown.

Analysis of the separate tissues showed both the epicarp and mesocarp to contain 800–900 mg./100 g. throughout the entire growing period (Fig. 4), whilst the endocarp gave much higher values (3400 and 2500 mg./100 g. in 1949 and 1950). These figures were obtained on the endocarp immediately prior to lignification. As this process proceeded, the concentration in the tissue as a whole showed a steady

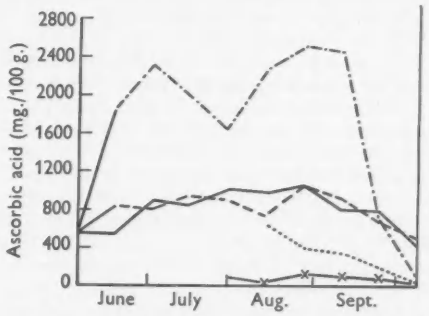

Fig. 4. Seasonal variation in concentration of ascorbic acid in the tissues of the fruit. ——, epicarp; — — —, mesocarp; —·—·—·, endocarp; ×—×, seed; . . . . . , shell.

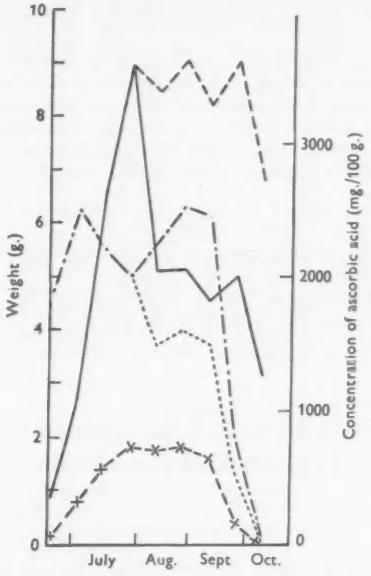

Fig. 5. The effect of lignification on the concentration of ascorbic acid in the endocarp of the fruit. ——, weight of soft tissue; — — —, weight of total tissue; —·—·—·, concentration of ascorbic acid in the soft tissue; . . . . . , concentration of ascorbic acid in the total tissue; ×—×, dehydroascorbic acid in the soft tissue.

decline to the fall of the fruit (Fig. 5). However, if the soft part of the tissue alone were considered, the concentration remained reasonably constant until the rapid decrease when maturation was complete.

This suggested that the high level was maintained only by virtue of the diminishing weight of the tissue.

Although surrounded by these extremely high concentrations, the seed (Fig. 4) showed very low levels, and the woody shell showed a steadily decreasing amount of ascorbic acid.

If the fruit is considered in conjunction with the rest of the plant, it is possible to follow the concentration gradient of ascorbic acid from the mesophyll of the leaves to the fruit stalk and possibly into the mesocarp. As the epicarp contains chlorophyll, it can supply further quantities of ascorbic acid by photosynthesis. These facts, however, do not explain the high levels in the endocarp which can be maintained by preformed ascorbic acid only if it is translocated against a steep concentration gradient, which is improbable (Wokes & Melville, 1948).

An alternative suggestion is that the ascorbic acid is synthesized in the endocarp. The intermediates in this proposed synthesis are first, precursors which are translocated from other parts of the plant, secondly, glucose (which is found in very high concentrations in the fruit), and thirdly, dehydroascorbic acid.

Zilva, Kidd & West (1938) showed that there was a change in the relative proportions of ascorbic and dehydroascorbic acids in Bramley's Seedling apples as the fruit developed, with the equilibrium shifting in favour of ascorbic acid. In the walnut, when the fruits are small, a concentration gradient of the oxidized form exists both from the outer tissues of the fruit and from the stem, into the endocarp.

It will be shown in a future paper, that in the presence of ascorbic acid, the epicarp and mesocarp are able to synthesize hydrojuglone glucoside. Examination of extracts failed to reveal sufficient dehydroascorbic acid to account for the high concentration of the glucoside in these tissues. It is presumed, therefore, that the dehydroascorbic acid is rapidly removed and utilized by the endocarp.

SUMMARY

1. A spectrophotometric assay for ascorbic acid and dehydroascorbic acid has been applied to extracts prepared from various parts of the walnut (*Juglans regia*).

2. Definite seasonal variations have been found for the concentration and amount per unit of these substances.

3. In order to explain the high concentration of ascorbic acid in the endocarp, which appears to preclude its translocation as such from the other parts of the plant, it is suggested that dehydroascorbic acid may be utilized by this tissue.

I wish to thank Dr F. Wokes, the Director of the Laboratories, for affording me the opportunity to publish this work, and Miss N. Baxter for technical help in the preparation of the many extracts used.

REFERENCES

Breinlich, J. (1942). *Süddtsch. Apoth. Ztg.* **82**, 195.
Daglish, C. (1950*a*). *Biochem. J.* **47**, 452.
Daglish, C. (1950*b*). *Biochem. J.* **47**, 458.
Daglish, C. (1951). *Biochem. J.* **49**, 635.
Gerghelezhiu, H. K. (1937). *Bull. appl. Bot. Genet. Plant Breeding, U.S.S.R.* Suppl. **84**, 2, 206.
Wokes, F. & Melville, R. (1948). *Biochem. J.* **43**, 585.
Zilva, S. S., Kidd, F. & West, C. (1938). *New Phytol.* **37**, 345.

Non-protein Nitrogenous Constituents of Rye Grass: Ionophoretic Fractionation and Isolation of a 'Bound Amino-acid' Fraction

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About one-sixth part of the organically combined nitrogen of freshly picked green leaves is in the form of compounds of low molecular weight, in the main free amino-acids. Since these are probably the most reactive nitrogenous compounds when leaves are eaten by the ruminant animal, or made into silage and so forth, it is important to have better knowledge of their nature, quite apart from the importance of this knowledge for plant physiology. During the latter part of the nineteenth century, studies of plant juices, especially those by Schulze, were very fruitful for biochemistry, and particularly the biochemistry of nitrogenous substances. Subsequently, however, such studies were rather neglected. Plant physiologists indeed made extensive series of measurements of the concentrations of individual compounds or groups of compounds, and their variation with changing conditions, but

there were few attempts at detailed characterization of the 'non-protein nitrogen' of particular plant tissues. Outstanding exceptions are Vickery's extended study of alfalfa constituents (Vickery, 1924*a*, *b*, 1925*a*, *b*; Vickery & Leavenworth, 1925; Vickery & Vinson, 1925) and a study of potato constituents by Neuberger & Sanger (1942; cf. Slack, 1948). In recent years, however, chromatographic methods have been used in a number of studies which have given a good general idea of the distribution of amino-acids in plant juices, confirming and extending the earlier observations, and in some cases providing rough quantitative data. Reference may be made to Allsopp (1948), Dent, Stepka & Steward (1947), Hulme & Arthington (1950), Joslyn & Stepka (1949), Steward, Thompson & Dent (1949), Westall (1950) and to a review by Steward & Thompson (1950).

In view of the difficulty of obtaining quantitative data on amino-acids from filter-paper chromatograms it has seemed useful to combine studies of this kind with quantitative fractionations by electrical-transport methods, and to base the amino-acid and peptide figures on the highly specific ninhydrin-carbon dioxide determination of Van Slyke and colleagues. In the present paper are described some data obtained in this way with rye grass. The results give a synopsis of the distribution of several categories of non-protein nitrogen in a typical forage material. There emerges a fairly detailed picture of the relative proportions of the main nitrogenous compounds when these figures are collated with the data from the filter-paper chromatograms, and with information about γ-aminobutyric acid (Synge, 1951). Since that paper was sent for publication, Beevers (1951) has demonstrated formation of γ-aminobutyric acid from L-glutamic acid by enzymic preparations from barley roots, and relatively large quantities have been found in mammalian brain by Awapara, Landua, Fuerst & Seale (1950) and by Roberts & Frankel (1950). These last authors produced evidence for its enzymic origin from glutamic acid, and their product was identified with authentic γ-aminobutyric acid by Udenfriend (1950), using an isotopic-labelling method.

Throughout the present work special attention has been paid to the recognition of possible peptide material ('bound' amino-acids). Such material has been reported in plant juices by a number of authors, but few have undertaken any detailed characterization. What has been learnt on this subject in the present work is discussed below in relation to these previous studies. Such substances may have importance as intermediates in the biological synthesis and breakdown of protein.

MATERIAL

Rye grass (*Lolium perenne* L.), strain S 23, was obtained through the kindness of Mr R. Bain from the grass plots of the North of Scotland College of Agriculture at Craibstone. The freshly picked material was treated with ether according to Chibnall (1939). It was hoped that this procedure, in which the cell walls are thought to function as dialysing membranes, would give a fairly representative mixture of the low-molecular components of the cell sap without allowing much opportunity for enzymic proteolysis and other changes that might occur on mincing or milling the tissues (cf. Tracey, 1948; Holden, Pirie & Tracey, 1950). At the same time possible alterations due to heating or drying (see Discussion) would be avoided.

The grass was placed in ether (anaesthetic, B.P.) for 20 min. The juice was then squeezed out in a hand press and filtered through paper pulp. Very different quantities of clear brown juice were obtained in this way from the grass in its various states, but the concentrations of components found in the juice did not vary strikingly. Nine samples of juice obtained on different dates from May to November had total N (Kjeldahl) in the range 0·51–1·33 mg./ml. The juice was placed in a cellophan sac and was dialysed against 4–5 vol. of $CHCl_3$ water for 96 hr. at 0–2° with occasional stirring. 72–94% of the N was diffusible under these conditions, assuming even distribution of diffusible material inside and outside the sac. All analyses given below are referred to the total N (Kjeldahl) of such diffusates. These were stored in the dark, saturated with $CHCl_3$, at 0–2°. No observations made during the necessarily protracted analyses suggested any changes in the nitrogenous compounds during storage. The pH of these diffusates was approx. 6. Three preparations specified in Table 1 were analysed in full detail.

METHODS

Ionophoretic fractionations

The first stage in the fractionation aimed at the separation of the non-protein nitrogenous substances into groups according to the net charge on the molecule at about pH 6, avoiding altogether the use of such empirical precipitants as lead acetate or phosphotungstic acid. Electrical-migration procedures seemed preferable to the various ion-exchange procedures that are available, since some of the compounds being studied were of unknown chemical nature and might be

Table 1. *Specimens of juice obtained from rye grass by the ether procedure*

Specimen	Hour of picking	Date of picking	N content of filtered juice before dialysis (mg. N/ml.)	Remarks
I	09.00	19. vi. 50	0·77	At flowering stage
II	14.00	1. ix. 50	0·73	Very dry; low yield of juice
III	—	30. xi. 49	1·08	Green and juicy; had been exposed to frosts

obtained in the wrong fraction or be irreversibly adsorbed during ion-exchange procedures.

The diaphragm-cell procedure of Theorell & Åkeson (1942) showed promise, particularly on account of their successful handling of glutamic and aspartic acids. By adding a special compartment for the anode, as in the electrolytic desalting apparatus of Consden, Gordon & Martin (1947), it was possible to collect these and other weak acids as a separate fraction without danger of them suffering anodic changes as in the original apparatus. The necessity for preliminary removal of chloride was thus also avoided. Fractionations of this kind may be of wide usefulness for biochemical separations, especially when weak acids are to be separated from bases, neutral substances and strong acids in a single operation. Work (1951; private communication) has evolved a similar arrangement for study of bacterial extracts and hydrolysates, and Sanger & Tuppy (1951) have made use of such a procedure in fractionating partial hydrolysates of insulin.

structed from Perspex sheet. Each compartment had approx. 50 ml. capacity. The details of construction and assembly are shown in Fig. 2. The membranes were held between rubber gaskets. The two terminal membranes were of cellophan, and the middle membrane of commercial sheepskin parchment which had been kept overnight at room temperature in a 1:5 (v/v) aqueous dilution of B.P. formalin and then washed thoroughly with distilled water. Since the higher current densities led to weakening and perforation of the parchment membrane, perhaps on account of local heating, a greater hole area was used for it than for the cellophan membranes. The platinum-wire electrodes were placed close to the terminal membranes as recommended by Theorell & Åkeson. The contents of each compartment were stirred by a slow stream of air or N_2. Cooling coils could be inserted as required into any compartment and temperatures were maintained at all times below 30°.

In operation, the cathode compartment was filled with 1% (w/v) aqueous NH_3; the solution for analysis was

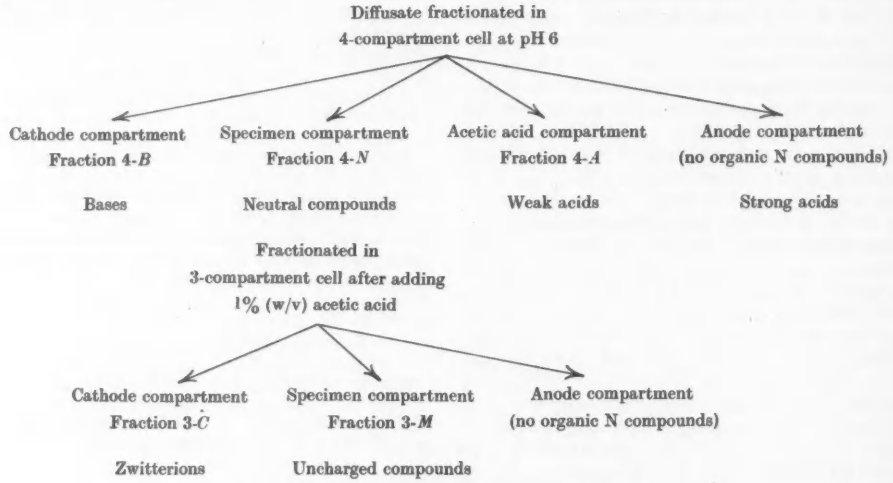

Fig. 1. Scheme of fractionation of grass-juice diffusates.

During preliminary study it was found that an important fraction of the 'bound' amino-acids stayed with the neutral amino-acids during fractionation in the four-compartment cell, but did not move with these into the cathode compartment when the mixture was acidified and subjected to ionophoresis as for the isolation of ω-amino-acids by Synge (1951), and of diketopiperazines by Gavrilov and colleagues (Gawrilow & Balabucha-Popzova, 1934; Balabucha-Popzowa, Gawrilow & Rikalewa, 1935; Gawrilow, Paradashvili, Balabouha-Popzova & Ljapounzowa, 1938; Balaboucha-Popsowa, Gawrilow, Paradachwili & Jakounine, 1938; Antonovich & Gavrilov, 1941). This observation formed the basis for obtaining these bound amino-acids free from other known N compounds by a second fractionation, this time in a three-compartment cell. The scheme of fractionation adopted is set out in Fig. 1.

Fractionation in four-compartment cell. The cell was of similar design to that of Theorell & Åkeson (1942), except that the compartments were open at the top. They were con-

adjusted to pH 6 and inserted in the next (specimen) compartment. The next (acetic acid) compartment was filled with 1% (v/v) aqueous acetic acid, to maintain its conductivity, and the anode compartment was filled with 0·1 N-H_2SO_4. 200 V. were then applied to the electrodes, though if much electrolyte was present such a lower voltage was applied in the early stages of the experiment as would keep the current below 200 ma. Where strong bases were present in the solution being analysed the current first rose as these entered the cathode compartment and increased its conductivity. The current then gradually fell as the specimen compartment was denuded of ions. The pH of the specimen compartment was checked repeatedly and maintained at pH 6 by additions of dilute NH_3 or acetic acid. Acetic acid was most often required in the early stages of a run and NH_3 later, but on many occasions the pH remained unchanged throughout. After 7 hr. the current had usually fallen to a constant value less than 5 ma., at which stage the fractionation was judged to be ended. Electroendosmosis took place into the cathode and

acetic acid compartments, but was not usually sufficient to cause overflowing or necessitate the withdrawal of solution. The contents of the cathode compartment could be removed at any stage and replaced with fresh aqueous NH_3 if it was desired to shorten the exposure of compounds which had migrated thither to the strongly alkaline conditions prevailing when salts had been present in the specimen. NH_3 was removed from the withdrawn solution by a short evaporation *in vacuo*, and then inorganic cations by subsequent treatment in the desalting apparatus of Consden *et al.* (1947). At the end of the run the contents of each compartment, with washings, were collected separately.

(aggregate recovery 101·0%). No Kjeldahl-reacting N was ever found in the anode compartment during any of the work described here. Presumably any nitrate present in the specimen migrated thither.

It was thus possible, by subjecting the contents of the cathode and acetic acid compartments to repeated fractionation, to obtain a very sharp quantitative separation of the basic, neutral and acidic components of the original mixture. For the purposes of the present exploratory work, however, a single fractionation only was used. The figures reported are without correction; the effect of correction would be to reduce still further the already low apparent quantities of

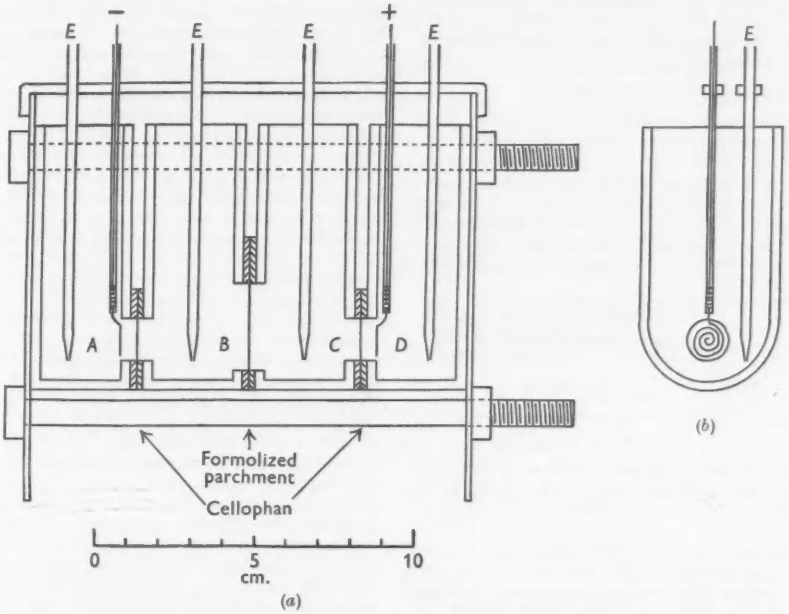

Fig. 2. Diaphragm cell assembled with four compartments: (*a*) sectional view from side. (*b*) end elevation of a compartment. *A*, cathode compartment; *B*, specimen compartment; *C*, acetic acid compartment; *D*, anode compartment; *E*, inlets for stirring gas.

On fractionating a mixture of alanine, ornithine and aspartic acid in this way the specimen compartment was found entirely free of ornithine and aspartic acid as judged by paper chromatography; ornithine with some contaminating alanine was found in the cathode compartment and aspartic acid, with a considerably smaller contamination by alanine, was found in the acetic acid compartment.

To obtain a quantitative picture of the amount of neutral material carried into the adjoining compartments a solution was made up to simulate crudely the non-nitrogenous dialysable constituents of a plant juice, with a single amino-acid, alanine, present: DL-alanine, 0·13; trisodium citrate dihydrate, 0·6; NaCl, 0·3; sucrose, 0·2% (w/v). At the end of the run, 3·8% of the alanine N was recovered in the cathode compartment (after removal of NH_3 by evaporation); 96·5% was found in the specimen compartment, 0·7% in the acetic acid compartment and nil in the anode compartment

basic amino-acids, but would otherwise not greatly affect the figures given.

As might be expected, histidine and the ω-amino-acids β-alanine and γ-aminobutyric acid were not always sharply separated into the cathode and specimen compartments respectively (cf. Sanger & Tuppy, 1951). However the filter-paper chromatograms showed very little histidine to be present and γ-aminobutyric acid and β-alanine made only small contributions to the ninhydrin-CO_2 figures (see below).

Fractionation in three-compartment cell. A three-compartment cell constructed as above was fitted with two cellophan membranes. The cathode compartment was filled with 1% (w/v) aqueous NH_3, the anode compartment with 0·1 N-H_2SO_4. In the middle compartment was placed a solution of fraction 4-*N*, to which had been added 1% (w/v) of acetic acid. On applying 200 V. the resulting current remained substantially constant (approx. 50 ma.), and at

the end of 7 hr. all free monoamino-acids had entered the cathode compartment (cf. Synge, 1951). The greater part of the bound amino-acids remained in the centre compartment together with much carbohydrate material.

Application of the ninhydrin-carbon dioxide determination to the study of plant extracts

Despite its high specificity for α-amino-acids little use has been made so far of the ninhydrin-carbon dioxide method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) for studies of plant material. Neuberger & Sanger (1942) used it for the determination of glutamine and other amino-acids in potato. The present work has shown that difficulties, so far not adequately explained, are encountered, but that nevertheless its usefulness is great.

As a routine, the determinations were done with 50 mg. triketohydrindene hydrate and citrate buffer (pH 4·7) at a vol. of 3 ml., as described by Van Slyke *et al.*, except that sealed evacuated hard-glass test tubes (6 × $\frac{5}{8}$ in.) were used as the reaction vessels. These were drawn with a shoulder (as shown in Fig. 3) so that they could be broken at a file scratch after connecting to the reaction chamber through a piece of pressure tubing (previously boiled out in dilute lactic acid).

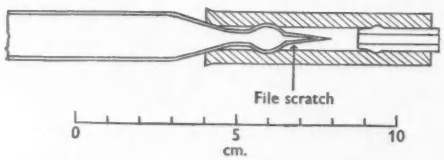

Fig. 3. Reaction tube connected to side arm of Van Slyke-Neill manometric apparatus for ninhydrin-CO_2 determination (before breaking off tip from tube).

The reaction chamber, side arm and tubing were evacuated before breaking the reaction vessel. The reaction was done at pH 4·7 rather than 2·5 to avoid, as far as possible, hydrolysis of peptides or CO_2 formation from uronides during heating under the more acid conditions. However, there was the disadvantage that at pH 4·7 γ-aminobutyric acid gave 0·13 mol. CO_2, whereas at pH 2·5 it gave no CO_2; Van Slyke *et al.* (1941) showed that β-alanine behaves similarly. These facts, as well as the yield of 2 mol. CO_2 from aspartic acid, should be borne in mind when interpreting the figures obtained.

Determinations were carried out on the original juices, and the fractions derived from them (*a*) without hydrolysis and (*b*) after hydrolysis for 24 hr. in 6 N-HCl at 105° in sealed evacuated tubes. The HCl hydrolysates were evaporated to dryness *in vacuo* before analysis, but no further attempts were made to adjust the pH, the amount of citrate buffer used being regarded as adequate. All determinations were in duplicate, about 0·1 mg. carboxyl N (Van Slyke *et al.* 1941, Table 1) being taken for each.

Determinations on unhydrolysed materials. When the plant materials were subjected to blank determinations without adding ninhydrin to the reaction mixtures considerable CO_2 was evolved. This was not due to dissolved CO_2, since prolonging the evaporation *in vacuo* before sealing the tubes did not diminish the amount evolved. Accordingly, all determinations on unhydrolysed material were done by measuring the difference of CO_2 evolution in the presence and absence of ninhydrin, samples being treated similarly in every other way. During the fractionations it was found that the material responsible for the blank CO_2 migrated into the cathode compartment; blank determinations on neutral and acidic fractions rarely differed much from the reagent blank. It was sometimes observed during the three-compartment stage of the fractionation that, while the material taken gave no blank, that recovered from the cathode compartment gave a considerable blank. The effects are tentatively attributed to carbamino compounds, either originally present or formed by interaction with atmospheric CO_2 under the conditions prevailing in the cathode compartment. In support of this view it was repeatedly observed, while analysing cathode-compartment contents from the four-compartment fractionations, that the blank CO_2 actually exceeded that evolved in the presence of ninhydrin, recalling the effect of ninhydrin in inhibiting the evolution of CO_2 from urea (Van Slyke *et al.* 1941). In any case this effect completely prevented calculation of carboxyl N in the unhydrolysed fractions 4-*B*, and introduced some error into the corresponding figures for fractions 3-*C* and for the unfractionated juices. Fractions from other compartments probably gave more nearly correct results, the small amounts of CO_2 evolved from uronides, ascorbic acid, etc., being allowed for by the blanks.

Determinations on HCl *hydrolysates.* When ninhydrin was omitted these never gave CO_2 differing significantly from the reagent blank, and blank determinations were accordingly not done in every case. Here the main source of error is that due to destruction of amino-acids during boiling with acid in the presence of carbohydrates. This has been discussed frequently in connexion with the hydrolysis of proteins. Vickery, Pucher, Wakeman & Leavenworth (1937) quote figures for amino N after hydrolysing edestin with 6 N-HCl in the presence of various carbohydrates. These show rather small losses. Tristram (1939) observed somewhat greater losses of basic amino-acids under similar conditions. Since the proportions of the more destructible amino-acids to total amino-acid residues are lower in the non-protein fractions than they are in common proteins, the errors here may be smaller still. At any rate the carboxyl-N figures for the acid hydrolysates of the various fractions on adding together agree rather well with the corresponding figures for unfractionated material, and it is felt that greater confidence can therefore be placed in these than in the carboxyl-N figures for unhydrolysed materials (see Table 2).

Determination of ammonia and amide N

Solutions were treated with at least 2 vol. of 10 N-HCl and kept at 37° for 10 days (cf. Gordon, Martin & Synge, 1941; Rees, 1946), after which the mixtures were evaporated at low temperature *in vacuo* and analysed for NH_3 according to Conway & O'Malley (1942), liberating the NH_3 with K_2CO_3. Free NH_3 was determined in the same way on the untreated solution, and the difference was recorded as amide N.

Determination of total N

This was done by a micro-Kjeldahl method. In view of the use of NH_3 in the cathode-compartment solutions, specimens from these were evaporated *in vacuo* at room temperature in the presence of excess NaOH before incineration.

Two-dimensional filter-paper chromatography of amino-acids

Chromatography was done by the ascending method (Williams & Kirby, 1948) successively with ammoniacal phenol-water and commercial collidine-water (Yorkshire Tar Distillers Ltd., Cleckheaton) using 35 cm. squares of Munktell OB paper (Grycksbo Pappersbruk AB, Grycksbo, Sweden). Samples usually contained 0·1 mg. N and were treated with H_2O_2 and ammonium vanadate on the paper (cf. Dent, 1948) to render visible methionine as the corresponding sulphone and cystine-cysteine as cysteic acid. The papers were dried below 40°, and the amino-acids were made visible by treatment with ninhydrin as usual, also below 40°; this was found to give the greatest differences between the colours given by the various amino-acids (cf. Dent, 1948).

Fractions containing salt or alkali were usually desalted according to Consden *et al.* (1947) before chromatography. Neutral fractions all gave excellent chromatograms without special treatment. The acidic fractions, owing to the presence of plant acids, often gave spots that were streaky in the phenol direction. Leucine and isoleucine were not resolved, and are not differentiated in what follows. In nearly all chromatograms studied there were sufficient of the well known amino-acids for ready identification simply from their relative positions and characteristic shades of colour in the early stages of reaction with ninhydrin. In doubtful cases mixed chromatograms with authentic amino-acids were run.

RESULTS

The quantitative data obtained in analyses of the diffusates from grass-juice preparations I, II and III (Table 1) are set out in Table 2. During fractionation in the four-compartment cell most of the orange-brown colouring matter of the diffusates tended to deposit itself on the parchment membrane; however, with some preparations, a bright yellow-green pigment entered the cathode compartment. The contents of the specimen and acetic acid compartments were usually pale yellow at the end of a run.

All the fractions were examined for ninhydrin-reacting substances by filter-paper chromatography before and after hydrolysis with 6N-hydrochloric acid for 24 hr. at its boiling point. Since the individual fractions gave better chromatograms than the unfractionated material, and since nothing was seen with the latter that was not seen with the former, only the observations on the fractions are described.

Fractions 4-B

Before hydrolysis. Arginine, lysine and γ-aminobutyric acid were always present, together with sometimes faint traces of histidine and of the two abundant neutral amino-acids alanine and glutamine.

After hydrolysis. The same, with additional very faint spots of nearly all the neutral and dicarboxylic amino-acids, among which leucine-isoleucine and valine were especially prominent.

Fractions 4-A

Before hydrolysis. Only glutamic and aspartic acids, with occasional faint traces of alanine and glutamine.

After hydrolysis. The same dicarboxylic acids; glutamine disappeared, alanine appeared or if previously present increased in intensity, and well defined, though not strong, spots due to phenylalanine, leucine-isoleucine, valine, β-alanine and glycine appeared. On one occasion a weak proline spot appeared.

Table 2. *Distribution of nitrogen in rye-grass juice diffusates and ionophoretic fractions therefrom*

(For methods of fractionation and determinations see text.)

	N as % of total N of diffusate from specimens (Table 1)		
	I	II	III
Unfractionated diffusate:			
Free ammonia	Nil	Nil	—
Amide N	28	22·4	—
Carboxyl N before hydrolysis	52·4	54	42
Carboxyl N after hydrolysis	54·5	58	48
γ-Aminobutyric acid (Synge, 1951)	—	—	6·2
Fraction 4-*B* (bases):			
Total N	25	38	25
Carboxyl N before hydrolysis	*	*	(1·9)*
Carboxyl N after hydrolysis	2·6	4·9	2·5
Fraction 4-*A* (weak acids):			
Total N	16·5	13	26
Amide N	Nil	2	—
Carboxyl N before hydrolysis	16·1	10·7	20·2
Carboxyl N after hydrolysis	15·9	11·3	21·6
Fraction 4-*N* (neutral substances):			
Total N	55	47	46
Amide N	11·8	10	—
Carboxyl N before hydrolysis	24·4	19·3	16·7
Carboxyl N after hydrolysis	39·6	32·4	24·6
Fraction 3-*C* (zwitterions):			
Total N	49·0	37·2	37·6
Carboxyl N before hydrolysis	19·6	23·6	21·8
Carboxyl N after hydrolysis	19·1	28·0	22·5
Fraction 3-*M* (uncharged substances):			
Total N	5·5	5·2	9·9
Amide N	1·0	1·3	—
Carboxyl N before hydrolysis	0·6	0·2	0·06
Carboxyl N after hydrolysis	9·0	3·7	5·2

* Not calculable (see text).

Fractions 4-N

Before hydrolysis. The following amino-acids were always seen: (strong) alanine, glutamine, γ-aminobutyric acid and serine; (medium) leucine-isoleucine, valine, proline; (weak) phenylalanine, tyrosine, β-alanine, glycine, threonine. Asparagine was variable, ranging from absent to medium; cysteic acid ranged

from absent to faint, and no indication of methionine was obtained.

After hydrolysis. Glutamic and aspartic acids appeared, glutamine and asparagine (if present) disappeared. No other new spots were seen, but the spots corresponding to leucine-isoleucine, valine and glycine nearly always showed obvious increases in intensity.

Fractions 3-C

Before hydrolysis. Same as 4-*N*.

After hydrolysis. Glutamine disappeared and glutamic acid appeared; if asparagine had been present it disappeared and aspartic acid appeared. Otherwise unchanged by hydrolysis.

Fractions 3-M

Before hydrolysis. Usually no material colouring with ninhydrin (nor was any colour seen during ninhydrin-carbon dioxide determinations on these fractions). Occasionally traces of aspartic acid were seen, which could be attributed to slight hydrolysis under the acid conditions of fractionation in the three-compartment cell (cf. Partridge & Davis, 1950).

After hydrolysis. Glutamic and aspartic acids, leucine-isoleucine, valine, alanine, proline and glycine appeared as spots of fairly equal intensities, although it was obvious that the relative proportions varied somewhat from preparation to preparation.

DISCUSSION

Free amino-acids

The free amino-acids found in the rye-grass diffusates are in general similar in nature and relative proportions to those noted by other workers with other plant juices (for references, see Introduction). Glutamine, alanine, serine and glutamic, aspartic and γ-aminobutyric acids predominate. In this connexion it is of interest that Vickery (1925*b*) isolated alanine and the then little-known serine from alfalfa juice. These two amino-acids also seem to be the first into which the carbon of newly assimilated carbon dioxide is incorporated by the plant (Calvin, Bassham & Benson, 1950; Vernon & Aronoff, 1950). Other common amino-acids are present in lower concentrations. Methionine and hydroxyproline were not seen. It has long been known that the proportions of glutamine and asparagine vary very much from species to species. It has more recently become apparent that the other free amino-acids of plants also differ more from species to species in their quantitative distribution than does the amino-acid composition of the tissue proteins (cf. Allsopp, 1948). Nevertheless, the data that have accumulated suggest a general similarity between the various species studied, and it is perhaps permissible to speak of a typical pattern of concentrations of free amino-acids in plant juices.

'Bound' amino-acids

Many workers with plant juices have referred to 'peptide nitrogen' and have made estimates of the amount present. Thus Vickery *et al.* (1937) found 0·05–0·26 g. 'peptide N'/kg. of fresh tobacco leaves. Such figures were obtained by measuring the increase in amino nitrogen on hydrolysing material which had been freed from protein by the use of heat or precipitants, and leave little doubt as to the existence of fair amounts of such non-precipitable non-heat-coagulable material. The ninhydrin-carbon dioxide data in the present work suggest that only a proportion of this is of such low molecular weight as to be able to pass through cellophan. Furthermore, in agreement with other workers referred to in the Introduction, in the present work no materials colouring with ninhydrin were seen on the chromatograms which disappeared to give amino-acids after acid hydrolysis, excepting of course the amides glutamine and asparagine. Simple open-chain peptides of low molecular weight are therefore not present in quantity.

Such peptides seem to occur in marine algae. Haas and colleagues have isolated from a number of seaweeds diffusible products having amino nitrogen and yielding on hydrolysis various amino-acids (Haas & Hill, 1931, 1933*a*, *b*; Haas, Hill & Russell-Wells, 1938; Haas, 1950). One such preparation, from *Pelvetia fastigiata*, has recently been studied in detail by Dekker, Stone & Fruton (1949), who tentatively formulated their product as L-pyrrolidonoyl-L-glutaminyl-L-glutamine, and thought that it might have arisen by lactam formation from L-glutaminyl-L-glutaminyl-L-glutamine during the isolation, which involved heat treatment. A peptide-like substance 'eisenine' was isolated by Ohira (1939, 1940*a*, *b*) from *Eisenia bicyclis*, using a similar procedure to that of Haas, and yielded on hydrolysis L-alanine, L-glutamic acid and ammonia. Ohira formulated eisenine as pyrrolidonoylglutaminylalanine. This conclusion has been criticized by Tazawa (1949). If peptides of this kind were present in rye-grass, they would be found in fraction 4-*A* if in the pyrrolidonoyl form or in fraction 3-*C* if in the glutaminyl form. However, the increase of carboxyl nitrogen on hydrolysing these fractions is slight.

Some positive conclusions can, however, be drawn as to the types of compound embodying bound amino-acids that occur in the rye-grass diffusates and these are set out below.

Basic compounds (fraction 4-B). Small amounts of a wide variety of neutral and dicarboxylic bound amino-acids occur in this fraction. These are therefore presumed to be linked to basic substances. Whether compounds are also present that yield basic but not other amino-acids on hydrolysis it is impossible to decide on the present evidence.

Vickery (1924*b*), using precipitation methods, obtained evidence for bound arginine and bound lysine in alfalfa juice, but, as already stated, there is no evidence that his materials were diffusible.

Acidic compounds (*fraction* 4-*A*). Judging from the ninhydrin-CO_2 figures for this fraction, only small quantities of bound amino-acids were present. As judged from the chromatograms, these consistently included phenylalanine, leucine-isoleucine, valine, alanine, β-alanine and glycine. Glutathione would be found in this fraction, and cannot therefore have been present in quantity. In addition to peptides, acyl derivatives should be considered as possibly responsible for the amino-acids liberated. Pantothenic acid is an obvious possible precursor of the β-alanine, and simpler acyl derivatives of amino-acids may occur (cf. Manske's (1937) isolation of δ-*N*-acetylornithine from *Corydalis ochotensis*).

Neutral compounds. The greater part of the bound amino-acids in the rye-grass diffusates stayed with the neutral amino-acids during fractionation in the four-compartment cell, and was recovered in fraction 4-*N*. It was unexpected to find that this material behaved as if devoid of ionizable groupings, a large part of it being recovered in fraction 3-*M* at the second stage of the fractionation. Some unaccounted losses of bound amino-acids occurred during this stage, if the carboxyl nitrogen figures are used as a basis of calculation, but the procedure used made it easy to isolate what is at least a substantial part of the bound amino-acids free from other known compounds of nitrogen, and the simplicity of the procedure should facilitate future studies.

This fraction accounts for 5–10 % of the nitrogen of the diffusate. Only a few different amino-acids are set free on acid hydrolysis. Their relative proportions differ somewhat from specimen to specimen. They are leucine-isoleucine, valine, proline, alanine, glycine and glutamic and aspartic acids. From the presence of amide nitrogen it may be inferred that these last occur as glutamine and asparagine residues. The compounds in question are, judging by their ionophoretic behaviour, devoid of ionizing groups, and this is confirmed, as concerns amino groups, by the absence of colour reaction with ninhydrin by the unhydrolysed material.

The possibility of these amino-acid residues being present as cyclic peptides (Synge, 1949; Bailey, 1951) is one that immediately suggests itself. However, since much carbohydrate (probably mostly glucose, fructose, sucrose and lower fructosans) is present in the same fraction, the possibility of the amino-acid residues being bound to carbohydrate is not excluded. Such complexes might conceivably arise under natural conditions by a non-enzymic reaction between free amino-acids and carbohydrates. However, to judge from the observations of Gottschalk & Partridge (1950), the amino-acids in question should not be very reactive with reducing sugars and the resulting compounds would possess ionizing groups. Nor would the conditions used during the isolation (avoidance of heat, high concentrations or alkaline pH) favour the formation of such compounds as artifacts (cf. Lea, 1950). Some of the diminution in amino nitrogen observed by Vickery *et al.* (1937, pp. 780–5) during the drying of tobacco leaves which had been exposed to light might well, however, be due to such a cause. These workers found that this amino nitrogen was liberated again on acid hydrolysis, and postulated interaction with some or other substances involved in the metabolism of carbohydrate.

The different categories of non-protein nitrogen

From the data presented here, taken with those on γ-aminobutyric acid (Synge, 1951), it is possible to drawn up a very rough quantitative expression of the distribution of the non-protein nitrogen of rye-grass (Table 3). There is a considerable non-amino-acid basic fraction, presumably containing such bases as choline and purines (cf. Vickery's work on alfalfa already cited and Neuberger & Sanger, 1942).

Table 3. *Approximate quantitative distribution of non-protein nitrogen of rye-grass diffusates*

	N as % of total N of diffusates
Non-amino-acid bases	15–25
Basic amino-acids, peptides, etc.	6–15
γ-Aminobutyric acid	5–10
Glutamine	20–25
Other free neutral amino-acids	10–15
'Bound' amino-acids in neutral compounds	4–10
Free glutamic and aspartic acids	10–20
'Bound' amino-acids in acidic compounds	1–3

Presumably any betaines occur in fractions 4-*N* and 3-*C*. Apart from these it is evident, from comparing the chromatographic data with the carboxyl-nitrogen figures, that most of the nitrogen is accounted for as well known amino-acids. The predominance of 'non-essential' amino-acids in this mixture readily explains the poor nutritional results when plant non-protein nitrogen is substituted for protein in non-ruminant animals (e.g. Henriques & Hansen, 1907; Slack, 1948). In fact, subsequent work has done little to modify views on this problem since Schulze (1908) appraised it in the light of his long analytical experience.

SUMMARY

1. A diaphragm-cell arrangement has been adapted for the quantitative ionophoretic fractionation of plant juices by adding an extra compartment to the apparatus of Theorell & Åkeson (1942). While it has been used here for separating nitrogen compounds according to the net charges on their molecules it should also prove useful for separations

of carbohydrates, mineral constituents, phosphoric esters, plant acids, etc.

2. The ninhydrin-carbon dioxide determination has been used for measuring free and 'bound' α-amino-acids in plant juices, and in this certain difficulties have been encountered.

3. The above techniques, together with qualitative two-dimensional filter-paper chromatography of amino-acids, have been used in studying those organic nitrogenous constituents of the juice of rye grass (*Lolium perenne*) which can diffuse through cellophan.

4. The bulk of this non-protein nitrogen was made up by basic substances and by well known amino-acids in the free state.

5. Small quantities of bound amino-acids were found in the form of basic and acidic compounds, but the greatest part of the bound amino-acids, representing 4–10 % of the diffusible non-protein nitrogen, was isolated ionophoretically in a fraction rich in carbohydrates but devoid of free amino-acids.

6. The material in this fraction seemed to be devoid of free amino or carboxyl groups, and on acid hydrolysis yielded ammonia, leucine and/or isoleucine, valine, proline, alanine, glycine, aspartic and glutamic acids.

7. The present observations are discussed in relation to previous work on the free and bound amino-acids of plant juices.

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REFERENCES

Allsopp, A. (1948). *Nature, Lond.*, **161**, 833.

Antonovich, E. G. & Gavrilov, N. I. (1941). *Zh. obshch. Khim.* **11**, 763. Cited in *Chem. Abstr.* (1942), **36**, 101.

Awapara, J., Landua, A. J., Fuerst, R. & Seale, B. (1950). *J. biol. Chem.* **187**, 35.

Bailey, K. (1951). *Biochem. J.* **49**, 23.

Balaboucha-Popsowa, W. S., Gawrilow, N. J., Paradachwili, A. J. & Jakounine, G. F. (1938). *Bull. Soc. chim. Fr.* [5], **5**, 978.

Balabucha-Popzowa, W. S., Gawrilow, N. I. & Rikalewa, A. M. (1935). *Biochem. Z.* **283**, 62.

Beevers, H. (1951). *Biochem. J.* **48**, 132.

Calvin, M., Bassham, J. A. & Benson, A. A. (1950). *Fed. Proc.* **9**, 524.

Chibnall, A. C. (1939). *Protein Metabolism in the Plant.* New Haven: Yale University Press.

Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.

Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.

Dekker, C. A., Stone, D. & Fruton, J. S. (1949). *J. biol. Chem.* **181**, 719.

Dent, C. E. (1948). *Biochem. J.* **43**, 169.

Dent, C. E., Stepka, W. & Steward, F. C. (1947). *Nature, Lond.*, **160**, 682.

Gawrilow, N. I. & Balabucha-Popzova, W. S. (1934). *Biochem. Z.* **271**, 292.

Gawrilow, N. I., Paradashvili, A. I., Balabouha-Popzova, W. S. & Ljapounzowa, S. W. (1938). *Bull. Soc. chim. Fr.* [5], **5**, 973.

Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.

Gottschalk, A. & Partridge, S. M. (1950). *Nature, Lond.*, **165**, 684.

Haas, P. (1950). *Biochem. J.* **46**, 503.

Haas, P. & Hill, T. G. (1931). *Biochem. J.* **25**, 1472.

Haas, P. & Hill, T. G. (1933*a*). *Ann. Bot., Lond.*, **47**, 55.

Haas, P. & Hill, T. G. (1933*b*). *Biochem. J.* **27**, 1801.

Haas, P., Hill, T. G. & Russell-Wells, B. (1938). *Biochem. J.* **32**, 2129.

Henriques, V. & Hansen, C. (1907). *Hoppe-Seyl. Z.* **54**, 169.

Holden, M., Pirie, N. W. & Tracey, M. V. (1950). *Biochem. J.* **47**, 399.

Hulme, A. C. & Arthington, W. (1950). *Nature, Lond.*, **165**, 716.

Joslyn, M. A. & Stepka, W. (1949). *Food Res.* **14**, 459.

Lea, C. H. (1950). *Chem. & Ind.* p. 155.

Manske, R. H. F. (1937). *Canad. J. Res.* **15** B, 84.

Neuberger, A. & Sanger, F. (1942). *Biochem. J.* **36**, 662.

Ohira, T. (1939). *J. agric. chem. Soc. Japan*, **15**, 370. Cited in *Chem. Abstr.* (1939), **33**, 6245.

Ohira, T. (1940*a*). *J. agric. chem. Soc. Japan*, **16**, 1; *Bull. agric. chem. Soc. Japan*, **16**, 10. Cited in *Chem. Abstr.* (1940), **34**, 3683.

Ohira, T. (1940*b*). *J. agric. chem. Soc. Japan*, **16**, 293; *Bull. agric. chem. Soc. Japan*, **16**, 71. Cited in *Chem. Abstr.* (1940), **34**, 5826.

Partridge, S. M. & Davis, H. F. (1950). *Nature, Lond.*, **165**, 62.

Rees, M. W. (1946). *Biochem. J.* **40**, 632.

Roberts, E. & Frankel, S. (1950). *J. biol. Chem.* **187**, 55.

Sanger, F. & Tuppy, H. (1951). *Biochem. J.* **49**, 463, 481.

Schulze, E. (1908). *Hoppe-Seyl. Z.* **57**, 67.

Slack, E. B. (1948). *Nature, Lond.*, **161**, 211.

Steward, F. C. & Thompson, J. F. (1950). *Ann. Rev. Plant Physiol.* **1**, 233.

Steward, F. C., Thompson, J. F. & Dent, C. E. (1949). *Science*, **110**, 439.

Synge, R. L. M. (1949). *Quart. Rev. Chem. Soc.* **3**, 245.

Synge, R. L. M. (1951). *Biochem. J.* **48**, 429.

Tazawa, Y. (1949). *Acta phytochim., Tokyo*, **15**, 141. Cited in *Chem. Abstr.* (1950), **44**, 3441.

Theorell, H. & Åkeson, Å. (1942). *Ark. Kemi Min. Geol.* **16** A, no. 8.

Tracey, M. V. (1948). *Biochem. J.* **42**, 281.

Tristram, G. R. (1939). *Biochem. J.* **33**, 1271.

Udenfriend, S. (1950). *J. biol. Chem.* **187**, 65.

Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.

Vernon, L. P. & Aronoff, S. (1950). *Arch. Biochem.* **29**, 179.

Vickery, H. B. (1924*a*). *J. biol. Chem.* **60**, 647.

Vickery, H. B. (1924*b*). *J. biol. Chem.* **61**, 117.

Vickery, H. B. (1925*a*). *J. biol. Chem.* **65**, 81.

Vickery, H. B. (1925*b*). *J. biol. Chem.* **65**, 657.

Vickery, H. B. & Leavenworth, C. S. (1925). *J. biol. Chem.* **63**, 579.

Vickery, H. B., Pucher, G. W., Wakeman, A. J. & Leavenworth, C. S. (1937). *Bull. Conn. agric. Exp. Sta.* no. 399.

Vickery, H. B. & Vinson, C. G. (1925). *J. biol. Chem.* **65**, 91.

Westall, R. G. (1950). *J. sci. Food & Agric.* **1**, 191.

Williams, R. J. & Kirby, H. (1948). *Science*, **107**, 481.

Work, E. (1951). *Biochem. J.* **49**, 17.

Paper Chromatography of Amines

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Although the method of paper chromatography has been employed successfully for the separation of amino-acids, sugars, purines, pyrimidines, phenolic compounds and other naturally occurring substances, relatively few attempts have so far been made to apply it to the separation and identification of amines. The behaviour of various primary aromatic amines on paper chromatograms has received some attention (Ekman, 1948; Tabone, Robert & Troestler, 1948; Tabone & Robert, 1948; Kelemen, Tanos & Halmagyi, 1950), but of the non-aromatic amines only the following groups of biologically important amines have been successfully separated: creatine, creatinine and their demethylated analogues (Maw, 1948; Ames & Risley, 1948); histamine and acetylhistamine (Urbach, 1949); adrenaline, noradrenaline and methyladrenaline (James, 1948); histamine, glucosamine, chondrosamine, choline, and ethanolamine (Dent, 1948; Partridge, 1948; Chargaff, Levine & Green, 1948; Aminoff, Morgan & Watkins, 1950).

The need for a rapid method of identifying small amounts of amines in biological materials became evident during the course of investigations on the metabolism of amino-acids in soil and in studies on plant amine oxidase. The results presented in this paper suggest that this problem can be solved by application of paper chromatography. The R_F values in various solvents of a large number of amines, including those of two homologous series (*n*-alkyl monoamines and polymethylene diamines), have been determined.

The results obtained with the two homologous series studied provided an opportunity to test the deduction (Martin, 1949) that the addition of a given group to a molecule will change its partition coefficient in a ratio depending only on the group and the phase pair concerned. From this it follows that the same ratio will relate the partition coefficients of all neighbouring pairs of a homologous series. Bate-Smith & Westall (1950) have recently shown that this deduction holds well enough for hydroxyl and glucose groups in a large number of phenolic substances. They tested the validity of the deduction by plotting $R_M \left[= \log_{10} \left(\frac{1}{R_F} - 1 \right) \right]$, which is a simple function of the partition coefficient and the constants of the chromatogram, against the number of groups and obtained series of approximately parallel straight lines for several homologous series. The same method was used in the present work.

METHODS

Apparatus. The apparatus used for determining the R_F values of the substances was similar to that described by Dent (1948). The only modification was that the lid of the cabinet had a small hole fitted with a rubber bung which could be removed to permit introduction of solvent into the trough.

Procedure. The amines were normally used in the form of 0·1 M aqueous solutions of their hydrochlorides. Where the hydrochlorides were not available aqueous solutions of the free bases were neutralized with HCl to pH 7·0 and diluted to 0·1 M. Agmatine was used in the form of an 0·1 M aqueous solution of its sulphate.

1–3 μl. of the amine solutions, containing 6–40 μg. of each amine, were applied at intervals of 3 cm. to a horizontal line ruled 6 cm. from the top of a large sheet of Whatman no. 4 filter paper. The papers were allowed to dry and then equilibrated overnight in the cabinet with the vapour of the aqueous phase of the solvent-water mixture to be used for irrigation. After this preliminary equilibration the solvent was added through the hole in the lid, and irrigation was continued until the leading edge of the solvent had travelled a distance of 35–45 cm. from the line of application. Depending on the solvent used the time required was 7–24 hr. The sheets were then removed from the cabinet, dried at room temperature or in an oven at 105°, sprayed with a 0·1 % (w/v) solution of ninhydrin in $CHCl_3$ and placed in an oven at 105° for 5 min. The positions of the solvent fronts were marked immediately after removal of the sheets from the cabinet. R_F values reported were determined at 16° ± 2°.

Solvents. (All percentages are v/v.) The solvents used were phenol, 'collidine', *n*-butanol, *n*-butanol-acetic acid and *m*-cresol-acetic acid. The 'collidine' mixture used was that described by Dent (1948), consisting of equal volumes of 2:4-lutidine and 2:4:6-collidine saturated with water. The *n*-butanol (40 %)-acetic acid (10 %)-water (50 %) mixture was that employed by Partridge (1948) for the separation of sugars. Before use this mixture was shaken thoroughly and allowed to stand for 3 days (Bate-Smith & Westall, 1950). The *m*-cresol (50 %)-acetic acid (2 %)-water (48 %) mixture used was that described by Bate-Smith & Westall (1950). Phenol runs were carried out both in the presence of NH_3 and HCN, and in an atmosphere saturated with 50 % aqueous acetic acid; 'collidine' runs were conducted in the presence of diethylamine (Dent, 1948).

RESULTS

R_F values of various amines in the solvents tested are recorded in Table 1. They were obtained with one batch of filter paper and represent the means of several determinations. In agreement with Bate-Smith (1949) the values were found to vary from batch to batch of filter paper, but to be reasonably constant within individual batches. Although the R_F values obtained with other batches of Whatman no. 4 paper were somewhat different, the degree of separation obtained was similar.

Small well defined spots were obtained using butanol or butanol-acetic acid as solvent; somewhat larger, elongated spots were obtained when phenol or *m*-cresol-acetic acid was used as solvent.

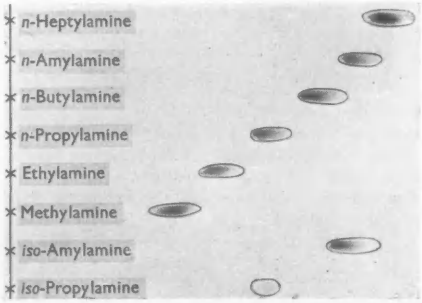

Fig. 1. Chromatogram on Whatman no. 4 filter paper of primary alkylamines run in butanol-acetic acid and sprayed with ninhydrin. Before photographing, the visible areas of the spots were outlined with pencil.

The tint and intensity of the colours obtained on spraying the chromatograms with ninhydrin were found to depend, to some extent, on the solvent used for irrigation. The colours were strongest when *m*-cresol-acetic acid was used as solvent and weakest when 'collidine' was employed. No attempt was made to determine the lower limit of sensitivity for the detection of each amine. However, the ninhydrin colour reaction seemed to occur as readily with the primary amines tested as with α-amino-acids, 10 μg. of most of the compounds being easily detectable. With the exception of ephedrine, the secondary amines tested gave much weaker colours with ninhydrin than did the primary amines. Trimethylamine, hordenine, choline and urea could not be detected with ninhydrin. Weak yellow spots of identical R_F value were obtained with ninhydrin when hydroxylamine and hydrazine were run in butanol (R_F 0·02) and butanol-acetic acid (R_F 0·12).

Butanol-acetic acid proved to be the most effective solvent for the separation of the primary *n*-alkylamines (see Fig. 1). The latter did not appear on chromatograms run in 'collidine', presumably owing to their loss by volatilization under the basic conditions employed.

Phenol alone and *m*-cresol-acetic acid were found to be the most satisfactory solvents for separating the polymethylene diamines.

It is obvious from Table 1 that useful separations of the other amines tested can be achieved by the choice of suitable solvents.

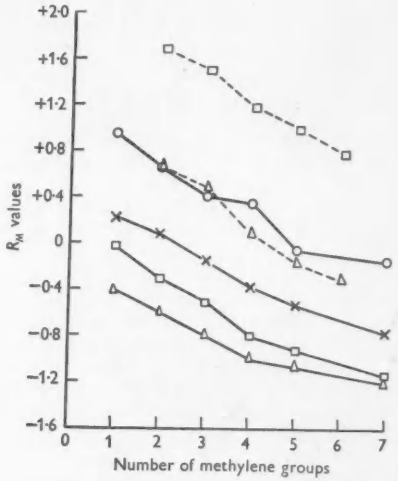

Fig. 2. $R_M\left[=\log_{10}\left(\frac{1}{R_F}-1\right)\right]$ values in various solvents of primary *n*-alkyl monoamines (——) and primary polymethylene diamines (– – – –). ○, *n*-butanol; ×, *n*-butanol-acetic acid; □, *m*-cresol-acetic acid; △, phenol. For details of solvent mixtures and experimental conditions see text.

The $R_M\left[=\log_{10}\left(\frac{1}{R_F}-1\right)\right]$ values for members of the two homologous series of amines studied in those solvents in which there were appreciable differences in R_F within the series are plotted against the number of methylene groups in Fig. 2. The results obtained for the diamines with phenol in an atmosphere of acetic acid are not included since, under the experimental conditions employed, the solvent-water mixture and the atmosphere may not have been in equilibrium. The curve in this case showed a sharp break.

DISCUSSION

The results presented above show that a large number of amines can be separated and identified by the paper-chromatographic technique. The use of ninhydrin for the detection of the amines studied again raises the question of the specificity of the

Table 1. *R_F values of amines in various solvents on Whatman no. 4 paper at room temperature*

Solvent (% v/v)	*n*-Butanol	*n*-Butanol (40%), acetic acid (10%), water (50%)	'Collidine'	*m*-Cresol (50%), acetic acid (2%), water (48%)	Phenol	Phenol	Phenol	—
Addition	—	—	Diethylamine	—	NH_3, HCN	Acetic acid	—	Colour with ninhydrin*
Methylamine	0·10	0·37	...†	0·52	0·96	0·91	0·72	*P*
Ethylamine	0·18	0·45	...†	0·67	0·95	0·94	0·80	*P*
n-Propylamine	0·28	0·58	...†	0·76	0·97	0·98	0·86	*P*
n-Butylamine	0·31	0·70	...†	0·86	0·96	0·98	0·91	*P*
n-Amylamine	0·53	0·77	...†	0·89	0·96	0·98	0·92	*P*
n-Heptylamine	0·58	0·85	...†	0·93	0·95	0·98	0·94	*P*
iso-Propylamine	0·27	0·57	...†	—	—	—	—	*P*
iso-Amylamine	0·52	0·77	...†	—	0·97	—	—	*P*
1:2-Diaminoethane	0·02	0·14	0·20	0·02	0·93	0·23	0·18	*P*
1:3-Diaminopropane	0·02	0·15	0·30	0·03	0·93	0·35	0·25	*P*
1:4-Diaminobutane (putrescine)	0·02	0·16	0·20	0·06 (0·38)‡	0·94	0·89	0·45	*P*
1:5-Diaminopentane (cadaverine)	0·02	0·17	0·22	0·09 (0·50)‡	0·94	0·94	0·59	*P*
1:6-Diaminohexane	0·03	0·20	0·24	0·14 (0·64)‡	0·94	0·97	0·67	*P*
Benzylamine	0·38	0·68	0·79	0·86	0·95	0·98	0·91	*RP*§
β-Phenylethylamine	0·51	0·72	0·80	0·88	0·95	0·97	0·91	*B*
ββ-Diphenylethylamine	0·50	0·72	0·80	0·86	0·94	0·97	0·90	*B*
β-Phenyl-β-hydroxyethylamine	0·35	0·65	0·79	0·84	0·94	0·96	0·86	*P*
Adrenaline	0·14	0·45	Streak	0·58	Streak	0·91	0·74	*P*‖
Agmatine	0·00	0·05	0·16	0·06 (0·64)‡	0·94	0·60 (0·93)‡	0·42 (0·75)‡	*P*
Allylamine	0·03	0·50	...†	0·14	0·95	0·95	0·86	*P*
Ethanolamine	0·09	0·33	0·44	0·47	0·90	0·85	0·65	*P*
Dimethylamine	0·12	0·43	...†	—	—	0·97	0·95	*P*‖
Ephedrine	0·53	0·75	0·86	0·95	0·94	0·96	0·94	*P*
Glucosamine	0·04	0·24	0·50	0·03	0·67	0·42	0·30	*P*
Histamine	0·03	0·19	0·44	0·09	0·96	0·94	0·68	*BG*
Spermine	0·00	0·07	0·10	0·02	0·93	0·33	0·24	*P*
Tryptamine	0·35	0·67	0·84	0·86	0·95	0·96	0·91	*GBr*
Tyramine	0·30	0·62	0·84	0·70	0·95	0·96	0·85	*GP*

* Final colours on chromatograms run in *n*-butanol-acetic acid: *P*, purple; *R*, red; *B*, blue; *G*, grey; *Br*, brown.
† These amines were not revealed by ninhydrin in this solvent.
‡ Values in brackets refer to weaker spot detected.
§ Colour initially yellow.
‖ Weak coloration with ninhydrin.

ninhydrin colour reaction. As Dent (1948) pointed out, there is still much confusion on this subject. He found that, contrary to the statement of Calvery (1944), β- and γ-amino-acids gave the reaction. He further showed that substances containing a primary aliphatic group, namely ethanolamine, glucosamine and histamine, gave colours with ninhydrin similar to those obtained with α-amino-acids. The results reported here indicate that this reaction is typical of compounds containing an aliphatic primary amino group. In addition, it appears that some compounds containing a secondary aliphatic amino group give the ninhydrin colour reaction. Tertiary amines do not appear to give the reaction.

Dent (1948) is of the opinion that only a limited number of ninhydrin-reacting substances other than α-amino-acids are likely to be present in mixtures obtained from natural sources. This is not borne out, however, by the recent work of Steward & Thompson (1950), who detected in plant materials some twenty ninhydrin-reacting substances which could not be identified with any of the known naturally occurring amino-acids. Several of these substances were found to be stable to acid hydrolysis and were, therefore, probably not peptides. The possibility that some of these acid-stable compounds were amines is suggested by the fact that several amines have recently been found in plant materials.

The chances of confusing amino-acids with amines when chromatographing mixtures of unknown composition would appear to depend largely upon the solvents employed for chromatography. Several of the solvents employed in this work have also found favour in paper-chromatographic analysis of amino-acid mixtures. The results obtained in this work show that in some of these solvents, e.g. butanol-acetic acid, many amines run at speeds similar to some of the naturally occurring amino-acids. However, under the conditions commonly employed for two-dimensional chromatographic analysis of amino-acid mixtures, i.e. using phenol-ammonia as first solvent and 'collidine' or butanol-acetic acid as second solvent, it would appear that only those amino-acids which have high R_F values in phenol-ammonia are likely to be confused with amines.

In this respect it may be pointed out that the chromatographic behaviour and properties of Dent's (1948) 'fast arginine'—believed to be a new basic amino-acid—are consistent with those of putrescine.

Although ninhydrin has been used for the detection of the amines employed in this work, it is obvious that there is much scope for the development of more specific methods of detection. For example, histamine, tyramine and tryptamine could be detected by the Pauly reagent, adrenalines by potassium ferricyanide (James, 1948) and agmatine by the Sakaguchi reaction.

Dent (1948) found that when phenol was used as solvent the R_F value of glucosamine in an atmosphere saturated with 1:1 (v/v) aqueous acetic acid was markedly smaller than the value obtained in an atmosphere of ammonia and hydrocyanic acid. We have confirmed the effect of acidity with this substance. However, it is clear from Table 1 that the majority of the amines tested were unaffected by this change of atmosphere, and that this test cannot be used to reveal the presence of amines in the mixtures under investigation.

It can be seen from Fig. 2 that when the R_M values in various solvents of the two homologous series of amines studied are plotted against the number of methylene (—CH_2—) groups a series of approximately parallel straight lines is obtained. It would appear, therefore, that Martin's (1949) deduction concerning the relationship between constitution and partition coefficient holds well enough for —CH_2— groups in primary *n*-alkyl monoamines and polymethylene diamines.

Finally, it may be pointed out that the findings in this work with regard to the effect of molecular structure on mobility in phenol are in agreement with those of other workers. For example, mobility is increased by decarboxylation (cf. R_F values of corresponding amino-acids) or substitution by a phenyl group of a hydrogen atom and decreased by replacement of a hydrogen atom by a hydroxyl group.

SUMMARY

1. The R_F values on filter-paper chromatograms of a large number of amines in various solvents have been determined.

2. Ninhydrin was found to be an effective reagent for the detection of primary aliphatic amines on paper chromatograms.

3. The results obtained suggest that amines likely to be present in biological materials could be separated and identified by paper chromatography.

4. Relationships between the molecular structures of the amines studied and their R_F values are discussed.

REFERENCES

Ames, S. R. & Risley, H. A. (1948). *Proc. Soc. exp. Biol., N.Y.*, **69**, 267.
Aminoff, D., Morgan, W. T. J. & Watkins, W. M. (1950). *Biochem. J.* **46**, 426.
Bate-Smith, E. C. (1949). *Biochem. Soc. Symp.* no. 3, 62.
Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. Biophys. Acta*, **4**, 427.
Calvery, H. O. (1944). *The Chemistry of the Amino Acids and Proteins*, ed. C. L. A. Schmidt, 2nd ed., p. 185. Springfield and Baltimore: Thomas.
Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Ekman, B. (1948). *Acta chem. scand.* **2**, 383.
James, W. O. (1948). *Nature, Lond.*, **161**, 851.
Kelemen, E., Tanos, B. & Halmagyi, D. (1950). *Biochem. J.* **47**, 138.
Martin, A. J. P. (1949). *Biochem. Soc. Symp.* no. 3, 4.
Maw, G. A. (1948). *Biochem. J.* **43**, 139.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Steward, F. C. & Thompson, J. F. (1950). *Ann. Rev. Plant Physiol.* **1**, 233.
Tabone, J. & Robert, D. (1948). *Ann. Inst. Pasteur*, **74**, 146.
Tabone, J., Robert, D. & Troestler, J. (1948). *Bull. Soc. chim. biol., Paris*, **30**, 547.
Urbach, K. F. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 146.

β-(3:4-Dihydroxyphenyl)ethylamine (hydroxytyramine) in Normal Human Urine

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Following the discovery by Holtz, Heise & Lüdtke (1938) of L-dopadecarboxylase in animal tissues, Holtz, Credner & Koepp (1942) demonstrated that after administration of 3:4-dihydroxyphenylalanine (DOPA) to man, guinea pigs, rabbits and cats, the urine contained considerable quantities of hydroxytyramine present in free and conjugated form. They also obtained some evidence for the presence normally of hydroxytyramine in urine. Holtz, Credner & Kroneberg (1947) later showed that normal urine contained what appeared to be a mixture of hydroxytyramine, adrenaline and noradrenaline (urosympathin). In a recent paper, however, Kroneberg & Schümann (1950) express the opinion that in man and certain animals noradrenaline forms most if not all of the catechol amines in urine.

The presence in normal urine of adrenaline and noradrenaline could be demonstrated biologically after adsorption on aluminium hydroxide (von Euler & Hellner, 1951 *a*, *b*). When purified urine extracts were submitted to paper chromatography, a mixture of *n*-butanol and N-hydrochloric acid being used as a solvent, not only noradrenaline and adrenaline were found, but also considerable quantities of another catechol amine occupying the site of hydroxytyramine on the chromatogram. The present paper deals with the separation and identification of hydroxytyramine from extracts of human urine by use of partition chromatography on a starch column.

EXPERIMENTAL

Preparation of extracts. Urine was obtained from medical students. The catechol amines were separated by adsorption in alumina in portions of 0·5–2 l. according to the technique described previously (von Euler & Hellner, 1951 *a*). In order to facilitate filtration of the alumina precipitate Hyflo Supercel (Johns Manville and Co.) was added when necessary in amounts of 30–50 g./l.

In two preparations smaller portions of whole urine were boiled for 20 min. at pH 2 in order to split conjugated catechol amines (von Euler & Hellner, 1951 *a*). This procedure generally increased the yield of biologically active catechol amines by 30–100%, presumably by splitting ethereal sulphates (Richter, 1940). The glucuronides (Clark, Akawie, Pogrund & Geissman, 1950) would presumably not be split by this mild hydrolysis, which, however, was chosen in order to minimize the inactivation of the free catechol amines (by racemization). No qualitative differences were observed between untreated and hydrolysed urine.

Partition chromatography on starch column. The catechol amines of the urine extract were taken up in *n*-butanol by repeated evaporation of the aqueous solution with *n*-butanol at low temperature *in vacuo*. The butanol extract thus obtained was suitably concentrated and used for partition chromatography on a starch column with *n*-butanol/N-HCl as solvent according to the technique used by Hamberg & von Euler (1950). Each 2 hr. sample was evaporated to dryness *in vacuo* and dissolved in 2 ml. of water. These solutions were used for colorimetric determination, paper chromatography and biological assay. A control run was made with a mixture of adrenaline, noradrenaline and hydroxytyramine, together with urine and the *R* values determined.

Colorimetric determination. Adrenaline and noradrenaline were estimated quantitatively by the colorimetric method of von Euler & Hamberg (1949). Hydroxytyramine was determined by essentially the same method, the iodine treatment being extended to 3 min. at pH 6·7.

Paper chromatography. Filter paper runs were made on Grycksbo OB paper with *n*-butanol/N-HCl as solvent for 20–30 hr. at 25°. The spots were developed with a weak solution of ferricyanide. The location of the spots was compared with those obtained with pure standard solutions.

Biological assay. The samples were assayed on the blood pressure of the cat and compared with values obtained using standard solutions. The different type of blood pressure effects produced by the three substances in question facilitated the identification. The different substances were localized by the *R* values and estimated colorimetrically. For comparison the hydrochlorides of the synthetic hydroxytyramine, (−)-noradrenaline and (−)-adrenaline were used.

Fig. 1. Paper chromatogram, normal human urine before hydrolysis. Solvent, *n*-butanol/N-HCl. 25 hr. at 25°. (1) Normal urine + noradrenaline and adrenaline. (2) Normal urine. (3) Noradrenaline, adrenaline and hydroxytyramine.

RESULTS

In a preliminary run the localization of noradrenaline, adrenaline and hydroxytyramine was ascertained by adding the pure substances to an extract of hydrolysed urine which had been transferred into butanol. The amounts added were rather higher than those estimated to occur in the amount of urine used for this experiment (about 2 l.), thereby allowing a definite localization.

The following *R* values were obtained: hydroxytyramine, 0·31; adrenaline, 0·25; noradrenaline, 0·16.

Results obtained with hydrolysed urine

Pooled urine was treated with acid as described above, and a butanol extract prepared. The primary extracts were readsorbed on alumina in order to obtain further purification, although this entailed some losses. Butanol solution of the urine extract (2 ml.) corresponding to 4·6 l. of urine was applied to the column. Colorimetric tests revealed two different groups of active samples with *R* values corresponding to hydroxytyramine and noradrenaline. The latter samples were subjected to paper chromatography and showed spots in the position typical of noradrenaline.

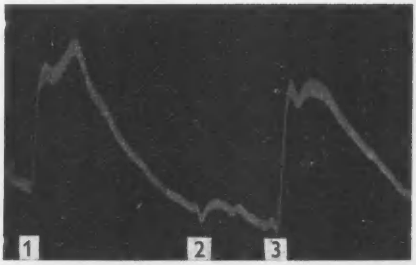

Fig. 2. Comparison of the effects on the blood pressure of the chloralosed cat of (1) authentic hydroxytyramine with (2) and (3). Material obtained from human urine. (1) 40 μg. hydroxytyramine (HOT). (2) Colorimetric equivalent of 5 μg. HOT (sample 26). (3) Colorimetric equivalent of 35 μg. HOT (sample 26).

As also observed on paper chromatograms with the urine extract, hydroxytyramine forms the largest fraction of the catechol amines (Fig. 1). The blood pressure response given by the 26 hr. sample showed a good agreement quantitatively and qualitatively with that given by authentic hydroxytyramine (Fig. 2). The total yield colorimetrically was 148 μg. estimated as hydroxytyramine.

Table 1. *R values and results of biological and colorimetric estimation of samples of catechol amines obtained from a starch column; first experiment*

(Hydrolysed material.)

Sample no.	Paper chromatography	Estimated		*R* value
		Colorimetrically	Biologically	
26	—	Equiv. to 100 μg. hydroxytyramine	Equiv. to 120 μg. hydroxytyramine	0·35
52–58	Noradrenaline	+(<20 μg.)	Noradrenaline	0·18

Table 2. *R values and results of biological and colorimetric estimation of fractions of urinary catechol amines obtained from a starch column; second experiment*

(Hydrolysed material.)

Sample no.	Paper chromatography	Estimated		*R* value
		Colorimetrically	Biologically	
26–28	Hydroxytyramine	108 μg. hydroxytyramine	Hydroxytyramine	0·36
34	—	—	Adrenaline	0·27
44–48	—	—	Noradrenaline	0·19

When repeated on a second preparation of urine obtained in the same way the results were the same as shown by the *R* values. In addition, evidence was obtained for the occurrence of adrenaline in this preparation.

Unhydrolysed urine

In order to ascertain whether urine contains hydroxytyramine in addition to adrenaline and noradrenaline in a preformed state, an experiment similar to those described above was undertaken with the difference that the urine was not boiled at pH 2 prior to the adsorption on alumina.

The results were essentially the same. Hydroxytyramine was found in a group of samples (*R* value, 0·36) and estimated colorimetrically to be 144 μg. In paper chromatograms adrenaline and noradrenaline were demonstrated in samples giving *R* values 0·28 and 0·19 respectively, i.e. in good agreement with the results obtained on hydrolysed urine.

DISCUSSION

The original claim of Holtz *et al.* (1942) that hydroxytyramine is normally excreted in urine has been confirmed. The doubts as to the normal occurrence of hydroxytyramine in normal human urine, which Kroneberg & Schümann (1950) recently expressed, have therefore not been substantiated. On the contrary, hydroxytyramine is, quantitatively, the most important catechol amine present. The earlier results of Holtz *et al.* (1942) that administration of DOPA leads to a considerable excretion of hydroxytyramine in urine gives indication that at least part of the hydroxytyramine found normally in the urine may be derived from DOPA by decarboxylation in the body.

As shown by Goodall hydroxytyramine occurs in sheep heart (1950*a*) and in suprarenal extracts (1950*b*), which is of interest since suprarenals may contain L-dopadecarboxylase in considerable amounts as shown by Langemann (1950). Goodall (1950*b*) also has obtained evidence by paper chromatography for the presence of DOPA in suprarenal extracts of thyroidectomized sheep. Bing (1940) has shown that hydroxytyramine is formed during perfusion of the isolated kidney with DOPA. In perfusates of isolated kidneys Williams & Grossman (1938) demonstrated the presence of a substance with the properties of hydroxytyramine. It is therefore not unlikely that the kidney, being rich in dopadecarboxylase, participates in the formation of hydroxytyramine.

The relatively large quantities of hydroxytyramine may interfere with the biological assay of other catechol amines in urine. The relative amount of hydroxytyramine may be estimated to be about five times that of noradrenaline. The pressor effect on the blood pressure of the cat is, therefore, still largely determined by noradrenaline, since this substance is fifty to hundred times more active than hydroxytyramine on the blood pressure of the cat. On the hen rectal caecum also the activity of hydroxytyramine is much less (10–20 times) than that of noradrenaline. Even if the hydroxytyramine content of the urine should amount to ten times that of noradrenaline, the apparent 'adrenaline' figure due to hydroxytyramine would not exceed 10% of the total catechols under the rather unfavourable assumption that the activity ratios of adrenaline and noradrenaline are as low as ten on the hen rectal caecum. As a rule the apparent 'adrenaline' figure caused by the hydroxytyramine would be less than 5% of the total catechol amines, and since the figures generally found in urine are of the order of 15–30%, the error will not be serious. On the other hand, if the noradrenaline figure is low and disturbances occur in the catechol amine distribution, the error may become appreciable.

SUMMARY

1. By partition chromatography on starch columns of human urine extracts hydroxytyramine, adrenaline and noradrenaline have been separated and identified.

2. The fractions with *R* value corresponding with those of hydroxytyramine give a characteristic spot in paper chromatograms and show the same behaviour as a hydroxytyramine on colorimetric estimation and biological assay.

3. Hydroxytyramine occurs largely in the free state in urine.

4. The daily output of hydroxytyramine is estimated at 0·1–0·2 mg. and exceeds considerably that of noradrenaline and adrenaline.

REFERENCES

Bing, R. J. (1940). *Amer. J. Physiol.* **132**, 497.
Clark, W. G., Akawie, R. I., Pogrund, R. S. & Geissman, T. A. (1950). *XVIIIth Int. physiol. Congr.* Abstr. p. 159.
Euler, U. S. von & Hamberg, U. (1949). *Science*, **110**, 561.
Euler, U. S. von & Hellner, S. (1951*a*). *Acta physiol. scand.* **22**, 161.
Euler, U. S. von & Hellner, S. (1951*b*). (To be published.)
Goodall, McCh. (1950*a*). *Nature, Lond.*, **166**, 738.
Goodall, McCh. (1950*b*). *Acta chem. scand.* **4**, 550.
Hamberg, U. & von Euler, U. S. (1950). *Acta chem. scand.* **4**, 1185.
Holtz, P., Credner, K. & Koepp, W. (1942). *Arch. exp. Path. Pharmak.* **200**, 356.
Holtz, P., Credner, K. & Kroneberg, G. (1947). *Arch. exp. Path. Pharmak.* **204**, 228.
Holtz, P., Heise, R. & Lüdtke, K. (1938). *Arch. exp. Path. Pharmak.* **191**, 87.
Kroneberg, G. & Schümann, H. J. (1950). *Arch. exp. Path. Pharmak.* **209**, 350.
Langemann, H. (1950). *XVIIIth Int. physiol. Congr.* Abstr. p. 325.
Richter, D. (1940). *J. Physiol.* **98**, 361.
Williams, J. R. & Grossman, E. B. (1938). *Amer. J. Physiol.* **123**, 364.

The Cytochrome System of Adrenal Medulla

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Huszak (1932, 1942) examined the cytochrome system of the medulla of the adrenal gland and reached the conclusion that, unlike all other animal tissues, it does not contain a cytochrome oxidase. The oxidation of its reduced cytochrome was said to be carried out by a peroxidase, together with hydrogen peroxide produced by the autoxidation of substances such as ascorbic acid or adrenaline. In the present investigation, however, it is shown that the adrenal medulla contains a perfectly normal cytochrome oxidase.

EXPERIMENTAL

Adrenal medulla preparation. Adrenal glands from ten or twelve horses at a time were obtained directly from the slaughter-house. The medulla (10 g. wet weight) was carefully cleaned from all traces of cortex, cut into small pieces about $5 \times 5 \times 1$ mm., and rapidly homogenized for 3 min. in a Waring blender with 100 ml. of cold 0·02 M-phosphate buffer, pH 7·3. The homogenate was then centrifuged at 2000 rev./min. for 10 min. and the supernatant was again centrifuged at 12,000 rev./min. for 25 min. The precipitate from the latter operation was then washed twice with 50 ml. of cold 0·01 M-phosphate buffer, pH 7·3, collected by centrifugation (12,000 rev./min., 25 min.) after each washing, and the final precipitate was suspended in an equal volume of 0·1 M-phosphate buffer, pH 7·3.

Cytochrome c was prepared by the method of Keilin & Hartree (1945) and contained 0·34% Fe.

Ferrocytochrome c was prepared immediately before use by reducing ferricytochrome *c* in 0·1 M-phosphate buffer, pH 7·3, with a current of hydrogen in presence of platinum black. Ferrocytochrome *c* thus prepared was slowly oxidized by the H_2O_2 formed in the solution from the Pt-catalysed reduction of dissolved O_2 by H_2. To prevent this oxidation, a few drops of dilute catalase solution were added immediately after the current of H_2 had been stopped. The Pt black was then removed by filtration.

Catalase was prepared by the method of Keilin & Hartree (1937).

Oxidation of ferrocytochrome c. The oxidation of ferrocytochrome *c* by the washed medulla preparation was followed by measuring the intensity of its α-band at 550 mμ. in a Beckman spectrophotometer. Each 1 cm. cell contained $1·7 \times 10^{-5}$ M-ferrocytochrome *c*; 0·1 M-phosphate buffer, pH 7·3; 0·1–0·2 mg. enzyme preparation; and 1 μg. catalase in a total volume of 3 ml. With gas mixtures other than air, 2 cm. cylindrical cells, provided with ground-glass stoppers, were used. These cells, with a total capacity of 5·5 ml., contained the same concentration of all the above reagents except that the cytochrome concentration was $0·85 \times 10^{-5}$ M. The required gas mixture was bubbled through the solution in these cells in presence of a drop of capryl alcohol before the addition of the enzyme. The concentration of ferrocytochrome *c* at time *t* is given by $(D_t - D_\infty)/(1·92 \times 10^4)$, where D_t and D_∞ are the density at time *t* and after complete oxidation respectively and $1·92 \times 10^4$ is the difference between the molecular extinction coefficients of reduced and oxidized cytochrome *c* at 550 mμ.

The activities of cytochrome oxidase, succinic dehydrogenase, succinic oxidase and dihydrocozymase oxidase were measured as described by Slater (1949*a*, *b*, 1950).

Peroxidase activity was measured either as described by Keilin & Mann (1937) or by a spectrophotometric method of Morell (1951).

RESULTS

Spectroscopic observations. Intact adrenal medulla tissue, as well as the washed preparation, showed a diffuse *d*-band of cytochrome at 530 mμ. and a

strong asymmetric *b*-band at 561 mμ., which was shaded towards the blue end of the spectrum. Unlike the cytochrome bands of heart muscle and kidney, the band at 561 mμ. remained in the reduced state despite thorough washing and vigorous shaking of the preparation in air. On the addition of a drop of a cytochrome *c* solution, the band at 561 mμ. was oxidized leaving a broad shading with its centre at about 556 mμ. If succinate was then added, both the cytochrome *b* originally present and the added cytochrome *c* were reduced. The oxidation as well as the reduction of cytochrome *b* in the adrenal medulla were very slow. The reduction could be hastened, however, by the addition of cyanide together with succinate. The medulla cytochrome *b* did not combine with carbon monoxide.

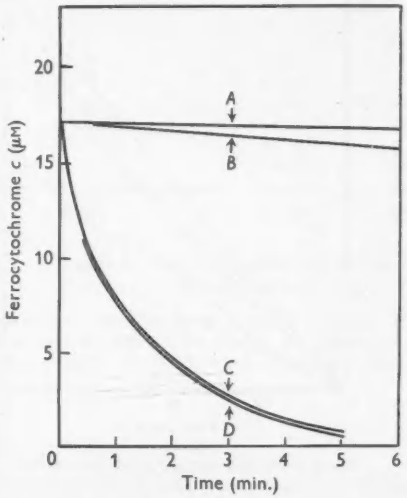

Fig. 1. Oxidation of ferrocytochrome *c*. Ferrocytochrome *c* was prepared by reduction with hydrogen in presence of Pt black. Curve *B* shows the slow oxidation by hydrogen peroxide formed from catalytic reduction of dissolved oxygen. In presence of catalase (curve *A*) there was no oxidation. Curves *C* and *D* show the oxidation of ferrocytochrome *c* by adrenal medulla preparation (0·31 mg.) in presence and absence of catalase respectively. Temp. 22°, 0·06 M-phosphate buffer, pH 7·3.

On cooling the washed preparation to the temperature of liquid air, a band was visible at 595 mμ. which is the position of the combined cytochromes *a* and a_3 band of heart-muscle preparation at this temperature (Keilin & Hartree, 1949). The band at 561 mμ. was moved towards the blue end of the spectrum and split into two bands at 552 and 558 mμ. respectively. The former band is not ascribed to cytochrome *c*, since at liquid air temperature this component is characterized by a main band at 547 mμ., together with a satellite band (Keilin & Hartree, 1949).

Cytochrome oxidase. The presence of a combined cytochrome *a* and a_3 band in adrenal medulla indicated that this tissue contained a cytochrome oxidase. Moreover, the adrenal medulla preparation catalysed the oxidation of ferrocytochrome *c* and the oxidation of ascorbic acid or *p*-phenylenediamine in presence of added cytochrome *c*.

Fig. 1 shows that in presence of the medulla preparation, ferrocytochrome *c* was rapidly oxidized by air. In addition, although ferrocytochrome *c* was very slowly oxidized by hydrogen peroxide, the rate of the rapid, medulla-catalysed oxidation was not affected by hydrogen peroxide which clearly indicates that the enzyme responsible for this catalysed

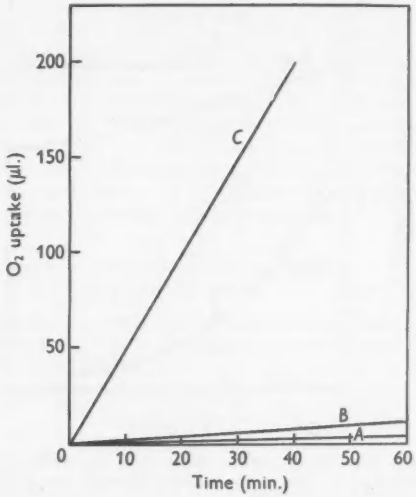

Fig. 2. Oxidation of *p*-phenylenediamine. O_2 uptake measured at 38°. Contents of the manometric flasks: 0·06 M-phosphate buffer, pH 7·3; 0·92 mg. fat-free dry wt. of adrenal medulla preparation; 50 μM-cytochrome *c*; 55 mM-*p*-phenylenediamine. Curve *A*, adrenal medulla preparation alone; curve *B*, as *A* with *p*-phenylenediamine; curve *C*, as *A* with *p*-phenylenediamine and cytochrome *c*.

oxidation of ferrocytochrome *c* is an oxidase and not a peroxidase.

The oxidation of *p*-phenylenediamine by the adrenal medulla preparation and added cytochrome *c* is shown in Fig. 2. The preparation alone or with added *p*-phenylenediamine gave no appreciable oxygen uptake, whereas a rapid oxygen uptake was obtained when both cytochrome *c* and *p*-phenylenediamine were added. Essentially the same results were obtained with ascorbic acid in place of *p*-phenylenediamine. The cytochrome oxidase activity

(Q_{O_2} expressed as μl. O_2/mg. dry wt./hr.) of different medulla preparations was usually between 400 and 600, as compared with 3000–3500 for heart-muscle preparation (Slater, 1949*a*).

Inhibition by carbon monoxide. Carbon monoxide inhibited both the oxidation of ferrocytochrome *c* by the preparation and the oxidation of ascorbic acid in presence of the preparation and cytochrome *c*. The former inhibition is shown in Fig. 3. It will also be noted that a change from air to a gas mixture of 95% N_2 and 5% O_2 did not affect the rate of oxidation of ferrocytochrome *c* catalysed by the preparation.

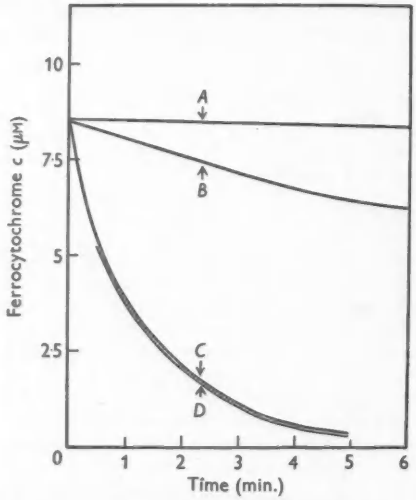

Fig. 3. Carbon monoxide inhibition of the oxidation of ferrocytochrome *c* by adrenal medulla preparation. Curve *A*, ferrocytochrome *c* alone; curves *B*, *C* and *D*, ferrocytochrome *c* in presence of adrenal medulla preparation (0·31 mg.). Curves *A* and *D*, air; curve *B*, 95% CO and 5% O_2; curve *C*, 95% N_2 and 5% O_2. Experimental conditions as for Fig. 1.

It has not been possible to demonstrate spectrophotometrically the reversal by light of the carbon monoxide inhibition of the oxidation of ferrocytochrome *c*. It is known that ferrocytochrome *c* shows strong absorption in the violet region of the spectrum, and being present in great excess over the oxidase it presumably acted as a filter which removed the greater part of the light at those wavelengths which are most effective in dissociating the oxidase-carbon monoxide complex. This is fully supported by the manometric experiments shown in Fig. 4. Under the experimental conditions of Fig. 4, the enzyme : cytochrome *c* ratio was increased 28-fold as compared with the experiments shown in Fig. 3, and irradiation by mercury-vapour light led to a considerable increase in the rate of oxygen uptake when carbon monoxide was present.

Effect of other inhibitors. The effect of various inhibitors on the oxidation of ascorbic acid by the medulla preparation and added cytochrome *c* is shown in Table 1. All the known inhibitors for haematin enzymes such as cyanide, azide, sulphide and hydroxylamine inhibited this reaction strongly. Diethyldithiocarbamate and phenylthiourea, both of which are powerful inhibitors for copper enzymes, did not inhibit this reaction. Fluoride had practically no effect.

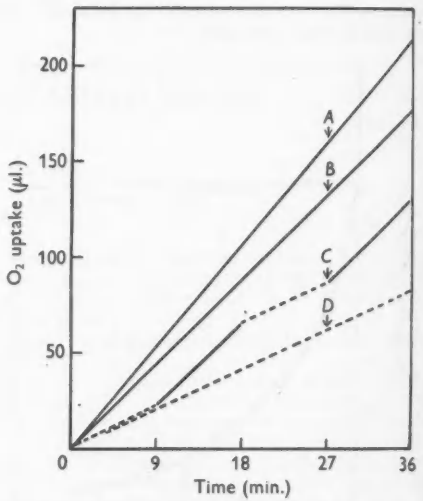

Fig. 4. Carbon monoxide inhibition of the oxidation of ascorbic acid in presence of adrenal medulla preparation and cytochrome *c* at 30°. Reaction mixture contained ascorbic acid, 0·026 M; $0{\cdot}7 \times 10^{-5}$ M-cytochrome *c*; 0·06 M-phosphate buffer, pH 7·3; and enzyme, 3·68 mg. fat-free dry wt. Curve *A*, in 95% N_2 and 5% O_2; curves *B*, *C* and *D*, in 95% CO and 5% O_2. For curves *B*, *C* and *D*: ——, reaction in light; - - - -, reaction in dark.

Oxidation of succinate and dihydrocozymase. The activities of succinic and dihydrocozymase oxidase systems in the medulla preparation are compared in Table 2 with those of heart-muscle and kidney preparations (Slater, 1949*b*, 1950). Like that of the heart-muscle and kidney preparations (Slater, 1950), the dihydrocozymase oxidase system of the medulla is also sensitive to BAL (2:3-dimercaptopropanol).

Although adrenal medulla has a strong cytochrome *b* band, it showed very little succinic dehydrogenase, whereas in most other tissues and micro-organisms, these two are usually closely associated (Table 3).

Table 1. *Effect of inhibitors on the oxidation of ascorbic acid by adrenal medulla preparation and added cytochrome* c

(Oxygen uptake measured at 38°. Reaction mixture contained 0·92 mg. fat-free dry wt. of enzyme; 0·026 M-ascorbic acid; 5×10^{-5} M-cytochrome *c*; 0·06 M-phosphate buffer, pH 7·3, and inhibitors as specified in the table. Percentage inhibition calculated on the basis of initial rates.)

Inhibitor	Concn. (M)	Inhibition (%)
Cyanide	5×10^{-7}	17
	5×10^{-6}	42
	5×10^{-5}	75
	5×10^{-4}	98
	5×10^{-3}	99
Sulphide	1×10^{-5}	62
	1×10^{-3}	87
Hydroxylamine	2×10^{-3}	32
	1×10^{-2}	55
	4×10^{-2}	72
Azide	2×10^{-5}	2
	2×10^{-4}	36
	2×10^{-3}	45
Fluoride	2×10^{-3}	0
	1×10^{-2}	5
Sodium diethyldithiocarbamate	3×10^{-3}	0
Phenylthiourea	3×10^{-3}	0

Peroxidase activity. Owing to the presence of a considerable amount of fat which interferes with the ether extraction, accurate determinations of peroxidase activity of the homogenate and of the washed preparation by Willstätter's method were impracticable. However, visual comparison of the purpurogallin colour formed in the reaction mixture with pyrogallol as substrate indicated that the 'Purpurogallinzahl' (P.Z.) (Willstätter & Stoll, 1918) of the homogenate was of the order of 0·001–0·01. Measured by a very sensitive spectrophotometric method (Morell, 1951), an alkaline phosphate extract, prepared exactly according to Huszak (1942), showed no peroxidase activity with pyrogallol as substrate.

DISCUSSION

One can postulate three possible mechanisms for the oxidation *in vivo* of cytochrome in the adrenal medulla: (1) a peroxidase acting in conjunction with hydrogen peroxide produced by the autoxidation of substances such as adrenaline; (2) a copper-protein enzyme; and (3) a haem-protein cytochrome oxidase, as in other tissues. These possibilities will now be examined in the light of the present study.

Table 2. *Succinic oxidase and dihydrocozymase oxidase activities of medulla preparation as compared with those of heart-muscle and kidney preparations*

(Succinic oxidase activity measured at 38° using the method described by Slater (1949*b*) except that the amount of enzyme used was 10·4 mg. fat-free dry wt. Dihydrocozymase activity determined at room temperature and calculated to 38°, assuming the same temperature coefficient as the heart-muscle enzyme (Slater, 1950).)

System	Added cytochrome *c* (M)	Activity of enzyme preparation (Q_{O_2})* Adrenal medulla	Heart muscle†	Kidney†
Succinic oxidase	—	1	500	60
	6×10^{-5}	30	650	200
Dihydrocozymase oxidase	—	—	870	50
	4×10^{-5}	80	1000	270

* Average values of different preparations.

† Figures taken from Slater (1949*b*, 1950).

Table 3. *Relationships between cytochrome* b *content and succinic dehydrogenase activity*

(The succinic dehydrogenase (Suc. D.) activity is expressed as Q_{O_2} measured in presence of methylene blue as described by Slater (1949*b*). Cytochrome *b* (Cyt. *b*) content was measured by comparison with a standard pyridine haemochromogen solution and expressed in μmol. cytochrome *b* haematin/g. fat-free dry wt. of preparation. Figures for heart-muscle and kidney preparations are taken from Slater (1949*b*).)

Preparation	Suc. D.	Cyt. *b*	Ratio Suc. D./ Cyt. *b*
Heart muscle	240	0·64	375
Kidney	67	0·27	248
Adrenal medulla	32	3·3	9·7

Peroxidase. It is clear that this tissue contains very little peroxidase activity. Moreover, whatever little peroxidase activity it has, can probably be accounted for by the presence of haemoglobin and cytochrome which are known to possess some peroxidase activity. The facts that (1) the rate of oxidation of ferrocytochrome *c* is not dependent on the presence of hydrogen peroxide, and (2) the catalysed oxidation of ascorbic acid in presence of the enzyme and added cytochrome *c* is inhibited by carbon monoxide definitely show that the oxidation of cytochrome is not carried out through a peroxidase.

Copper-protein enzyme. It has been claimed that a copper-protein enzyme, laccase, is capable of

catalysing the oxidation of cytochrome *c* (Bertrand, Belval & Legrand, 1947), although this has not been confirmed by Tissières (1949). In the present case, however, the possibility that a copper-enzyme is involved does not appear to be likely since two powerful copper-enzyme inhibitors, phenylthiourea and diethyldithiocarbamate, do not affect the action of the enzyme and, in addition, the carbon monoxide inhibition is light sensitive.

Cytochrome oxidase. All the experimental data brought forward in this paper lead to the conclusion that the enzyme responsible for the oxidation of cytochrome in adrenal medulla, as in all other animal tissues so far studied, is a haem-protein cytochrome oxidase. This view is supported by the facts that: (1) at the temperature of liquid air a combined band of cytochromes *a* and a_3 can clearly be seen; (2) the enzyme is affected to the same degree as cytochrome oxidase of other animal tissues by all known inhibitors of cytochrome oxidase; (3) the enzyme functions as well at low oxygen partial pressure as in air; and (4) it is inhibited by carbon monoxide and this inhibition is reversed by light.

Other cytochrome components. That the adrenal medulla contains very little, if any, cytochrome *c* is revealed by spectroscopic observations at the temperature of liquid air. The most prominent band of the medulla cytochrome system is that of cytochrome *b* at 561 mμ. (Bierich & Rosenbohm, 1928; Huszak, 1942), although Cohen & Elvehjem (1934) reported that the adrenal medulla contained cytochrome *c* but no cytochrome *b*. The presence in this tissue of an enzyme capable of catalysing the oxidation of ferrocytochrome *c* seems to suggest that cytochrome *c* is also present, but at such a low concentration that it cannot be detected by spectroscopic observation even at the temperature of liquid air. A point of some interest is that although this tissue shows a strong cytochrome *b* band, it has very little succinic dehydrogenase activity. This is in agreement with the view (Tsou, 1951) that succinic dehydrogenase is not identical with cytochrome *b* as was suggested by some workers.

SUMMARY

1. Spectroscopic observation showed the presence in adrenal medulla preparation of a strong cytochrome *b* band at 561 mμ. Cooling to the temperature of liquid air, which shifts all absorption bands towards the shorter wavelengths, further revealed a weak band at 595 mμ. corresponding to the combined band of cytochromes *a* and a_3.

2. Adrenal medulla preparations catalysed the oxidation of ferrocytochrome *c* and the oxidation of ascorbic acid and *p*-phenylenediamine in presence of added cytochrome *c*.

3. This catalytic oxidation of ascorbic acid was inhibited by cyanide, azide, sulphide, hydroxylamine and carbon monoxide, the latter inhibition being light reversible.

4. It is concluded that the adrenal medulla, like all the other animal tissues, contains the usual cytochrome system, though the cytochrome *c* content is very low.

5. The adrenal medulla contained very little, if any, peroxidase activity with pyrogallol as substrate.

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REFERENCES

Bertrand, D., Belval, H. & Legrand, G. (1947). *Bull. Soc. Chim. biol., Paris*, **27**, 608.
Bierich, R. & Rosenbohm, A. (1928). *Hoppe-Seyl. Z.* **178**, 250.
Cohen, P. P. & Elvehjem, C. A. (1934). *J. biol. Chem.* **107**, 97.
Huszak, I. (1932). *Biochem. Z.* **252**, 397.
Huszak, I. (1942). *Biochem. Z.* **312**, 330.
Keilin, D. & Hartree, E. F. (1937). *Proc. roy. Soc.* B, **121**, 173.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 282.
Keilin, D. & Hartree, E. F. (1949). *Nature, Lond.*, **164**, 254.
Keilin, D. & Mann, T. (1937). *Proc. roy. Soc.* B, **122**, 119.
Morell, D. B. (1951). Private communication.
Slater, E. C. (1949*a*). *Biochem. J.* **44**, 305.
Slater, E. C. (1949*b*). *Biochem. J.* **45**, 1.
Slater, E. C. (1950). *Biochem. J.* **46**, 484.
Tissières, A. (1949). *Nature, Lond.*, **163**, 480.
Tsou, C. L. (1951). *Biochem. J.* **48**, lii.
Willstätter, R. & Stoll, A. (1918). *Liebigs Ann.* **416**, 21.

Further Observations on the *in vitro* Stimulation by Insulin of Fat Synthesis by Lactating Mammary Gland Slices

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The incorporation of [*carboxy*-^{14}C] acetate into fatty acids by rat-liver slices *in vitro* was studied by Bloch & Kramer (1948), who showed that the rate of incorporation was increased by pyruvate and still further enhanced if insulin was present as well. The insulin effect was, however, not seen if glucose replaced pyruvate, nor did it occur in acetate alone. Later, Bloch (1948) reported that these results could not be reproduced regularly with other strains of rats, and he concluded that further work was necessary before the general significance of this insulin effect, believed to be particularly related to the utilization of pyruvate for fatty acid synthesis, could be evaluated.

Following upon this work we were able, by using lactating mammary gland tissue of the rat, which is an eminently favourable medium for the study of *in vitro* fat synthesis (Folley & French, 1949*a*, 1950), to demonstrate a marked stimulating effect of insulin upon *in vitro* fat synthesis in this tissue in mixtures of acetate and glucose, as judged by respiratory data and acetate uptake (Balmain, French & Folley, 1950). A definite but less marked effect was observed in glucose alone. Our results thus differ in certain respects from those of Bloch & Kramer (1948) as do the recent results of Brady & Gurin (1950), who have reported that insulin increases the incorporation of ^{14}C into long-chain fatty acids by rat-liver slices in presence of methyl- or carboxyl-labelled acetate alone, but not in acetate plus pyruvate.

In this paper we report further observations upon the stimulating action of insulin *in vitro* on fat synthesis by lactating mammary gland slices. The phenomenon has now been studied in mammary tissue from the mouse, rabbit and ewe, as well as more extensively in tissue from the rat, especially in relation to the functional state of the mammary gland. The important question of the mechanism of this remarkable *in vitro* insulin effect has also been considered in a preliminary way. The active mammary gland normally secretes large quantities of neutral fat in the form of glycerides, and it would therefore appear that the problem of the net fat synthesis which mammary gland slices are clearly capable of effecting *in vitro* under suitable conditions (Folley & French, 1950) is essentially a problem of glyceride formation rather than merely of fatty acid synthesis. That being so, the question of the origin of the glycerol must be taken into consideration, and the possibility of the availability of glycerol being critical for the synthesis of neutral fat, and at one remove of fatty acids, in mammary tissue, naturally arises. In view of the possibility that the stimulating action of insulin on fat synthesis in this tissue, demonstrated by the present work, might, partly at any rate, be connected with the regulation of the supply of glycerol, we have studied the effects of the addition of glycerol on the respiratory metabolism and acetate uptake of lactating mammary tissue. Some of this work has already been communicated in preliminary form (Balmain & Folley, 1951).

EXPERIMENTAL

The experiments were carried out on hooded Norway rats, fawn mice (Glaxo FF strain), Dutch rabbits and sheep, only animals which were lactating satisfactorily being used, except when tissue taken at the end of lactation was required. As regards laboratory species, abdominal mammary glands were always used for slicing, which was carried out as before (Folley & French, 1949*a*). Respiratory measurements were made by the method of Dickens & Simer (1931), most experiments lasting 2 hr., but some 3 hr. Quotients ($-Q_{O_2}$, Q_{acid}, $Q_{glucose}$) are expressed throughout as μl./mg. final dry wt./hr. Experimental details were as described previously (Folley & French, 1950), $-Q_{acid}$ being taken as an approximate measure of acetate uptake, since acetate was the only consumable anion used as substrate. In all cases, respiratory measurements in the presence and absence of insulin were made on tissue from the same gland. In one series of experiments residual glucose was determined in the medium at the end of the 2 hr. manometric run by the method of Nelson (1944).

'Regular' insulin was used in the experiments on rabbits, but for all the others a preparation of crystalline insulin (Novo), assaying approximately 20 i.u./mg. and free from glycogenolytic factor was used. The insulin was dissolved in the water to a concentration of 0·5 mg./ml. using the least possible amount of NaOH. The final pH of the solution was 7·4 (glass electrode).

RESULTS

In vitro *effect of insulin on fully lactating mammary tissue*

Rat. Results for four series of experiments in glucose plus acetate on tissue from rats in full lactation, thirty-seven animals in all, are summarized in Table 1. In all these experiments the substrate

concentrations were: sodium acetate 0·02 M; glucose, 0·3 %. This concentration of glucose was used because it had previously been found, by measurement of the time curve of total gas exchange, that while the pressure increase, due to excess production of carbon dioxide over oxygen uptake, soon fell off in 0·1 % glucose, it was approximately linear over at least 3 hr. when the initial glucose concentration was 0·3 % (Balmain *et al.* 1950).

It will be seen that in all four series $-Q_{O_2}$, $-Q_{acid}$ (≏acetate utilization) and R.Q. were significantly increased by addition of 1 i.u. insulin/ml. In the last column but one are given the values for 'extra CO_2', i.e. the CO_2 produced in excess of that required for an R.Q. of unity (the algebraic sum of Q_{CO_2} and $-Q_{O_2}$), the value for complete oxidation of either substrate. In this system this quantity may be taken as a rough indication of the extent of the occurrence of synthetic reactions leading to the formation of oxygen-poor substances (e.g. fatty acids) from oxygen-rich materials (carbohydrate, acetate). From the figures in the last column it will be seen that in all four series insulin increased the 'extra CO_2' production 2·5–3·0 times. Not only was the increase in $-Q_{O_2}$ and R.Q. accompanied by an increase in acetate utilization as indicated by a two- to three-fold increase in $-Q_{acid}$ but, as Table 2 shows, insulin also nearly doubled the glucose utilization.

Table 1. *Effect of insulin on the respiration and acetate utilization of lactating rat mammary gland slices in glucose* (0·3 %) *plus sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml. In this and succeeding tables the values given are means ± S.E.M.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of respiration experiments (hr.)	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	Increase in 'extra CO_2' due to insulin (%)
1	10	12–18	3	Insulin	13·4±0·9	−4·7±0·6	2·03±0·02	13·7±1·0	149
				None	10·5±0·9	−2·1±0·3	1·53±0·03	5·5±0·6	
2	11	14–15	2	Insulin	14·7±0·8	−4·8±0·5	2·15±0·02	16·8±0·7	195
				None	10·1±0·5	−1·4±0·2	1·57±0·04	5·7±0·5	
3	10	13–15	2	Insulin	11·7±0·6	−2·5±0·2	2·07±0·03	12·5±0·8	178
				None	8·6±0·6	−0·7±0·2	1·53±0·05	4·5±0·5	
4	6	14–16	2	Insulin	9·7±0·9	−1·7±0·5	1·89±0·03	8·6±0·7	161
				None	8·6±0·5	−0·5±0·2	1·40±0·04	3·3±0·4	

Table 2. *Effect of insulin on the glucose uptake of lactating rat mammary gland slices in glucose* (0·3 %) *plus sodium acetate* (0·02 M)

(Residual glucose in medium determined by the method of Nelson (1944) at end of respiration experiment.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of experiment (hr.)	Addition	$-Q_{glucose}$
3	7	13–15	2	Insulin	9·2±0·9
				None	4·7±0·8

Table 3. *Effect of insulin on the respiration and glycolysis of lactating rat mammary gland slices in glucose* (0·3 %)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of respiration experiments (hr.)	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	Increase in 'extra CO_2' due to insulin (%)
1	10	12–18	3	Insulin	10·5*±0·7	1·7±0·3	1·80±0·02	8·2±0·5	52
				None	9·2*±0·7	1·7±0·4	1·57±0·03	5·4±0·6	
2	11	14–15	2	Insulin	11·0†±0·7	3·2±0·2	1·87±0·04	9·3±0·7	79
				None	9·4†±0·6	3·3±0·3	1·55±0·04	5·2±0·4	

* Statistical analysis of these 10 pairs of differences gave $0·1 > P > 0·05$.
† Statistical analysis of these 11 pairs of differences gave $0·05 > P > 0·04$.

The results of two series of experiments carried out with 0·3 % glucose alone as substrate are given in Table 3. Here again R.Q. was significantly increased in the presence of insulin, but not to the same extent as when acetate was also present. The mean values of $-Q_{O_2}$ were greater in presence of insulin than in its absence, the difference in series 1 being significant at a level of probability between 5 and 10 % and in series 2 at a level just below 5 %. In glucose alone the 'extra CO_2' increased by 60–85 % as compared with 146 and 191 % in the same two series of experiments with glucose plus acetate as substrate.

In the presence of acetate alone, which is hardly utilized by rat mammary gland slices (Folley & French, 1950), insulin had no effect on the respiration of the tissue under the conditions of these experiments (Table 4).

Rabbit. Results for seven experiments on mammary gland slices from lactating rabbits (3–5 weeks) are given in Table 5. These were early experiments in which 'regular' insulin was used and a few of them have been previously reported (Balmain *et al.* 1950). They were carried out before it was found that a glucose concentration of 0·3 % was necessary for the full expression of the insulin effect, at any rate in experiments with rat mammary tissue. The substrate concentrations used in these experiments were: glucose, 0·1 %; acetate, 0·02 M, and the duration of all but one experiment was 3 hr. Even under these possibly suboptimal conditions insulin in all cases caused marked increases in R.Q., oxygen uptake and acetate utilization. The 'extra CO_2' production was substantially raised by insulin in all experiments.

Mouse. The mean results for an experiment on a group of five lactating mice (14 days in lactation) are given in Table 6. In glucose alone insulin had no appreciable effect on the respiration of mouse mammary gland slices, the R.Q. being of the same order as found previously for mouse tissue (Folley & French, 1949*a*), irrespective of whether or not insulin was present. In the absence of insulin, addition of acetate to the medium appreciably

Table 4. *Effect of insulin on the respiration and acetate utilization of lactating rat mammary gland slices in sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Experimental series	No. of rats	Stage of lactation at autopsy (days)	Duration of respiration experiments (hr.)	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.
1	10	12–18	3	Insulin	4·6±0·4	−0·5±0·2	0·82±0·04
				None	4·6±0·3	−0·2±0·1	0·76±0·03
2	3	14–15	2	Insulin	4·6	−0·2	0·82
				None	5·1	+0·8	0·75

Table 5. *Effect of insulin on respiration and acetate utilization of lactating rabbit mammary gland slices in glucose* (0·1 %) *plus sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95 % O_2, 5 % CO_2; temperature: 37°; 'regular' insulin (Wellcome) added to final concentration of 1 i.u./ml.)

Rabbit no.	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	$\Delta -Q_{O_2}$	ΔQ_{acid}	Δ R.Q.	Δ 'Extra CO_2'	Increase in 'extra CO_2' due to insulin (%)
101	Insulin	11·7	− 9·1	1·66	7·7	1·1	−2·8	0·46	5·6	267
	None	10·6	− 6·3	1·20	2·1					
C4	Insulin	12·3	− 9·2	1·63	7·8	2·2	−3·2	0·35	5·0	179
	None	10·1	− 6·0	1·28	2·8					
C5	Insulin	9·1	− 7·3	1·72	6·6	0·8	−2·9	0·49	4·7	247
	None	8·3	− 4·4	1·23	1·9					
108	Insulin	12·3	− 9·4	1·52	6·4	1·1	−3·6	0·31	4·0	167
	None	11·2	− 5·8	1·21	2·4					
114	Insulin	9·8	− 6·8	1·47	4·6	1·6	−2·3	0·30	3·2	229
	None	8·2	− 4·5	1·17	1·4					
116*	Insulin	19·6	−17·6	1·56	11·0	6·5	−8·3	0·28	7·3	197
	None	13·1	− 9·3	1·28	3·7					

* 1 hr. experiment; all others 3 hr.

lowered the R.Q. as compared with the results obtained in glucose, and Q_{acid} was still positive though somewhat smaller than in glucose alone, indicating possibly a slight utilization of acetate. The effect of glucose on acetate utilization by mouse mammary tissue is thus much less marked than that found previously in the rat and rabbit (Folley & French, 1950). Under these conditions, however, the mouse tissue was now responsive to insulin which restored Q_{O_2} and R.Q. to levels similar to those obtained in glucose alone and definitely increased the acetate uptake. In fact there is an indication that in glucose plus acetate the 'extra CO_2' production in presence of insulin was somewhat above the level found in glucose alone.

Sheep. Three lactating ewes were used for this study, biopsy specimens being taken from their udders, under *cyclo*propane anaesthesia, at various times from days 11–48 of lactation. Because of this it was considered advisable to subject the results to an analysis of variance which, however, revealed no regular trends with advance of lactation. Mean values in acetate are therefore given in Table 11 and in acetate plus glucose in Table 12. It is clear that in neither substrate had insulin any significant effect on the metabolism of the slices. The stimulatory effect of glucose on acetate utilization by udder slices from lactating ewes, previously reported (Folley & French, 1950), is confirmed by the present results (cf. Tables 11 and 12).

Insulin sensitivity of rat mammary tissue at different functional stages

The effect of insulin on the respiration of mammary tissue was studied on tissue from groups of rats killed at the end of pregnancy (19–20 days), at the height of lactation (14–15 days) and 2 days after the removal of the young at the 20th–21st day of lactation. The results obtained in glucose + acetate are given in Table 7. As found previously (Folley & French, 1949*b*), for tissue from rats of the same colony metabolizing glucose, the respiration of tissue taken at the end of pregnancy was relatively low, and the R.Q. below unity. Addition of insulin had no effect at this stage in contrast with the usual marked effect observed with fully lactating tissue (14–15 days). Similarly, after 2 days' weaning, low values for Q_{O_2} and R.Q. were found as before, and the slices no longer responded to insulin. Essentially similar changes in insulin sensitivity at the three stages studied were found in glucose alone (Table 8).

Comparison of the effects of glycerol and insulin on the respiration of lactating mammary gland slices

The results of 2 hr. determinations in glucose plus acetate on tissue from ten lactating rats, killed at days 13–15, are summarized in Table 9. It will be seen that glycerol in 0·2% concentration, like insulin, increases R.Q., $-Q_{O_2}$ and acetate utilization,

Table 6. *Effect of insulin on the respiration and acid utilization or production of lactating mouse mammary gland slices in glucose* (0·3%) *plus sodium acetate* (0·02M) *or in glucose* (0·3%) *respectively*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium, Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; duration of experiments: 2 hr.; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml. Five mice at the 14th day of lactation were used.)

	Glucose				Glucose + acetate			
Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)
Insulin	9·8±1·1	3·1±0·4	2·14±0·07	10·7±0·9	12·1±0·4	−1·0±0·7	2·14±0·08	13·7±1·3
None	10·6±0·8	2·4±0·2	1·90±0·07	9·5±0·9	10·1±0·3	0·1±0·3	1·46±0·05	4·4±0·2

Table 7. *Insulin sensitivity in glucose* (0·3%) *plus sodium acetate* (0·02M) *of mammary gland slices from rats at the end of pregnancy, mid-lactation and after weaning at* 20–21 *days*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Stage	No. of rats	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' ($Q_{CO_2}+Q_{O_2}$)
Pregnancy (19–20 days)	11	Insulin	1·4±0·1	0·1±0·04	0·90±0·03	—
		None	1·4±0·1	0·2±0·03	0·90±0·05	—
			Δ 0·0	Δ −0·1	Δ 0·00	—
Lactation (14–15 days)	11	Insulin	14·7±0·8	−4·8±0·5	2·15±0·02	16·8±0·7
		None	10·1±0·5	−1·4±0·2	1·57±0·04	5·7±0·5
			Δ 4·6	Δ −3·4	Δ 0·58	Δ 11·1
Post-weaning (2 days)	10	Insulin	4·5±0·2	2·7±0·2	0·81±0·04	—
		None	4·6±0·4	2·8±0·2	0·81±0·04	—
			Δ −0·1	Δ −0·1	Δ 0·00	—

Table 8. *Insulin sensitivity in glucose (0·3 %) of mammary gland slices from rats at the end of pregnancy, mid-lactation and after weaning at 20–21 days*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.)

Stage	No. of rats	Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' $(Q_{CO_2}+Q_{O_2})$
Pregnancy (19–20 days)	11	Insulin	1·3±0·1	0·8±0·1	0·85±0·03	—
		None	1·1±0·1	0·9±0·04	0·74±0·04	—
			Δ 0·2	Δ −0·1	Δ 0·11	—
Lactation (14–15 days)	11	Insulin	11·0±0·7	3·2±0·2	1·87±0·04	9·3±0·7
		None	9·4±0·6	3·3±0·3	1·55±0·04	5·2±0·4
			Δ 1·6	Δ −0·1	Δ 0·32	Δ 4·1
Post-weaning (2 days)	10	Insulin	4·5±0·2	3·3±0·1	0·80±0·03	—
		None	3·4±0·3	2·9±0·2	0·78±0·05	—
			Δ 1·1	Δ 0·4	Δ 0·02	—

Table 9. *Comparison of the effects of insulin and glycerol on the respiration and glucose and acetate utilization of lactating rat mammary gland slices in glucose (0·3 %) plus sodium acetate (0·02 M)*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; duration of experiments, 2 hr.; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2%. Ten rats (13–15 days of lactation) used for respiration measurements; glucose determinations carried out on seven of these.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' $(Q_{CO_2}+Q_{O_2})$	$-Q_{glucose}$
None	8·6±0·6	−0·7±0·2	1·53±0·05	4·5±0·5	4·7±0·8
Insulin	11·7±0·6	−2·5±0·2	2·07±0·03	12·5±0·8	9·1±0·9
Glycerol	10·9±0·7	−3·0±0·5	1·70±0·03	7·8±0·7	4·2±0·8

though under the conditions used the increase in R.Q. and 'extra CO_2' was less with glycerol than with insulin. It is noteworthy that glycerol differed from insulin in that it evoked no increase in glucose

Table 10. *Lack of additive effect of insulin and glycerol on the respiration and acid utilization of lactating rat mammary gland slices in glucose (0·3 %) plus sodium acetate (0·02 M)*

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2%. The values given are means of three experiments carried out on rats at the 14th–15th day of lactation.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2' $(Q_{CO_2}+Q_{O_2})$
None	6·9	−0·3	1·63	4·3
Insulin	10·0	−2·5	2·19	11·9
Glycerol	9·7	−2·5	1·76	7·5
Insulin + glycerol	11·4	−4·0	2·07	12·5

utilization. Table 10 shows that the effects of insulin and glycerol were not additive. Control experiments showed that glycerol alone was not utilized by rat mammary gland slices.

Since mammary gland slices from ruminants, in contrast with tissue from non-ruminants such as the rat, will utilize acetate alone with R.Q. > 1 (Folley & French, 1950), it was felt that a comparison of the *in vitro* effects of glycerol and insulin on mammary tissue from lactating ruminants would be of interest. The studies on lactating udder tissue from ewes, referred to above, therefore included an investigation of the effects of glycerol. The results (Table 11) indicate that while lactating sheep udder tissue is inert to insulin in acetate, the slices respond to glycerol by noticeable increases in $-Q_{O_2}$, R.Q. and acetate utilization which, though not dramatic, are nevertheless highly significant statistically. In glucose plus acetate (Table 12), on the other hand, glycerol appeared to cause a slight inhibition of fat synthesis as manifested by small but significant decreases in $-Q_{O_2}$, R.Q. and acetate utilization.

DISCUSSION

There is little doubt that the utilization of acetate or glucose with R.Q. > 1 by lactating mammary gland slices signifies the net synthesis of fat in the tissue. This conclusion, reached by Folley & French (1950), has been reinforced as far as the incorporation of acetate carbon into fatty acids is concerned by the

Table 11. *Comparison of the effects of insulin and glycerol on the respiration and acetate utilization of udder slices from lactating ewes in sodium acetate* (0·02 M)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; duration of experiments, 2 hr.; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2%. Fourteen biopsy samples were taken from three sheep at various times between days 11–48 of lactation.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2'* ($Q_{CO_2}+Q_{O_2}$)
None	5·1±0·3	−3·1±0·4	1·09±0·04	0·4
Insulin	5·4±0·3	−3·4±0·4	1·09±0·04	0·5
Glycerol	6·7±0·3	−5·6±0·4	1·27±0·04	1·8
	Significance of treatment comparisons			
Insulin—no addition	$P>0·05$	$P>0·05$	$P>0·05$	—
Glycerol—no addition	$0·01>P>0·001$	$P<0·001$	$P<0·001$	—

* Calculated from the mean values of $-Q_{O_2}$ and R.Q.

Table 12. *Comparison of the effects of insulin and glycerol on the respiration and acetate utilization of udder slices from lactating ewes in sodium acetate* (0·02 M) *plus glucose* (0·3%)

(Respiratory data and Q_{acid} measured by the method of Dickens & Šimer (1931); medium: Krebs-bicarbonate-Ringer solution, pH 7·4; gas phase: 95% O_2, 5% CO_2; temperature: 37°; duration of experiments, 2 hr; crystalline insulin (Novo) added to final concentration of 1 i.u. (0·05 mg.)/ml.; final concentration of glycerol, 0·2%. Fourteen biopsy samples were taken from three sheep at various times between days 11–48 of lactation.)

Addition	$-Q_{O_2}$	Q_{acid}	R.Q.	'Extra CO_2'* ($Q_{CO_2}+Q_{O_2}$)
None	7·6±0·3	−6·4±0·4	1·52±0·04	4·0
Insulin	8·0±0·3	−6·5±0·4	1·50±0·04	4·0
Glycerol	6·4±0·3	−4·3±0·4	1·42±0·04	2·7
	Significance of treatment comparisons			
Insulin—no addition	$P>0·05$	$P>0·05$	$P>0·05$	—
No addition—glycerol	$0·02>P>0·01$	$0·01>P>0·001$	$0·05>P>0·02$	—

* Calculated from the mean values of $-Q_{O_2}$ and R.Q.

demonstration that the mammary gland of the pregnant rabbit (Popják & Beeckmans, 1949, 1950), lactating goat (Popják, French & Folley, 1950, 1951) and lactating cow (Kleiber, Smith & Tolbert, 1950) actively incorporates $CH_3{}^{14}COOH$ into fatty acids of milk glycerides. It follows, therefore, that the *in vitro* insulin effect on the metabolism of lactating mammary gland slices, described in this and the preceding paper (Balmain *et al.* 1950), is an expression of a stimulation of net fat synthesis by the tissue. While an insulin concentration of 0·05 mg. (1 i.u.)/ml. has been uniformly used in the present work, in unpublished experiments we have been able to detect, by measurement of overall pressure changes (see Balmain *et al.* 1950), definite *in vitro* effects of insulin on lactating rat mammary tissue at concentrations as low as 0·0001 mg. (0·002 i.u.)/ml.

The fact that the effect we have studied is of such a magnitude as to be regularly demonstrable by respiratory measurements is no doubt due to the fortunate circumstance that in mammary tissue we have at our disposal a particularly favourable medium for the study of fat synthesis *in vitro*. This may be largely because this tissue is capable of effecting the synthesis and secretion of glycerides, a process which by 'fixing' fatty acids and removing them from the site of synthesis and breakdown would tend to favour their synthesis. Though the liver can synthesize fatty acids, it may be that their assembly into glycerides is not so important a feature of the fat metabolism of isolated liver tissue. If so, and if the tentative suggestion (see below) that the action of insulin on fat formation might be partly concerned with the regulation of the supply of glycerol for glyceride synthesis should prove correct, it would be understandable that *in vitro* insulin effects on fat synthesis from acetate in liver slices have so far only been demonstrated by increased incorporation of labelled carbon into fatty acids, which may signify merely an increased rate of fatty acid turnover rather than net synthesis.

The general significance of our results as regards the role of insulin as a regulator of fat synthesis in the mammary gland is emphasized by the fact that the insulin effect has been observed, under suitable conditions, in tissue from the rabbit and mouse as well as from the rat. Mammary tissue from lactating ewes was, however, inert to insulin both in acetate alone and in glucose plus acetate. This may be another facet of the metabolic difference between mammary tissue from ruminants and non-ruminants described by Folley & French (1950). With rat

mammary tissue the same insulin effect, but of lesser magnitude, was obtained as before (Balmain *et al.* 1950) in glucose alone, while mouse mammary gland was unresponsive to insulin with this substrate. Insulin had no effect on the metabolism of rat (or sheep) mammary tissue in presence of acetate alone, a result which differs from those of Brady & Gurin (1950), who studied the incorporation of ^{14}C-labelled acetate into fatty acids of rat-liver slices. Though the insulin effect studied by us is manifest in glucose alone (rat) but not in acetate alone (sheep and rat), it nevertheless seems particularly related to acetate utilization for fat synthesis. This is illustrated in Table 13, which shows that the enhancing effect of addition of acetate on fat synthesis by rat mammary gland slices metabolizing glucose is greatly increased in presence of insulin.

deposition by rat diaphragm muscle (Gemmill & Hamman, 1941), an effect which is antagonized by the anterior pituitary, as indicated by the results of Stadie, Haugaard & Marsh (1951), who found rat diaphragm to be more sensitive to insulin after hypophysectomy of the donor.

The important question of the mechanism of the insulin effect on fat synthesis must be largely a matter of speculation at the present time, but the fact that glycerol is capable of similarly stimulating fat synthesis by mammary gland slices may be significant in this connexion. It was previously suggested (Folley & French, 1949*c*, 1950) that the stimulating effect of glucose on fat synthesis from acetate by mammary gland slices might be explained, at least partly, on the assumption that glucose provides glycerol necessary for the formation of

Table 13. *Acetate metabolism of lactating rat mammary gland slices in relation to insulin*

(The Δ values given are differences between mean values in glucose (0·3%) plus acetate (0·02M) and in glucose (0·3%).)

Experimental series	Addition	(Glucose + acetate − glucose) $\Delta - Q_{O_2}$	ΔQ_{acid}	Δ R.Q.	Δ 'Extra CO_2'
1	Insulin	2·9	−6·4	0·23	5·5
	None	1·3	−3·8	−0·04	0·1
2	Insulin	3·7	−8·0	0·28	7·5
	None	0·7	−4·7	0·02	0·5

The inertness of rat mammary tissue to insulin at the end of pregnancy or after weaning parallels the low oxygen uptake and R.Q. at these stages previously observed in glucose (Folley & French, 1949*b*), and now confirmed both in glucose and glucose plus acetate. It would seem that insulin by itself cannot stimulate fat synthesis by fully developed but essentially non-functional mammary tissue, though this may appear surprising in view of recent evidence that the mammary gland of the rabbit in late pregnancy is capable of the synthesis of short-chain fatty acids characteristically found in milk fat (Popják & Beeckmans, 1949, 1950; Popják, Folley & French, 1949). The effect of insulin on the fat metabolism of mammary tissue seems to require for its expression the existence of an active secretory condition, manifested by the active respiration with high R.Q. exhibited by post-partum tissue (Folley & French, 1949*b*) and presumably related to the action of the complex of anterior-pituitary hormones concerned in lactogenesis and galactopoiesis (Folley & Young, 1941). The loss of insulin sensitivity following weaning emphasizes the probable importance of the regularly applied suckling stimulus, acting on the anterior pituitary (Selye, 1934), in maintaining the conditions essential for responsiveness to insulin. In respect of this evident necessity for anterior-pituitary participation the insulin effect studied by us differs from the well known stimulating action of insulin on glucose uptake and glycogen glycerides which, as pointed out above, might well indirectly promote fatty acid synthesis in a tissue capable of synthesizing and secreting glycerides. This suggestion has received support from the recent demonstration by French & Popják (1951) that radioactive carbon is incorporated into glycerol as well as fatty acids isolated from milk glycerides secreted by rabbits receiving ^{14}C-labelled glucose, which led them to conclude that glycerol might be a limiting factor in fatty acid synthesis. Our results with glycerol thus open up the possibility that insulin, acting at some point in the metabolic pathway of glucose breakdown, might promote the formation of glycerol from glucose. Two of our findings are in keeping with this hypothesis: (*a*) though R.Q. and acetate utilization of rat mammary gland slices is increased by glycerol in presence of glucose plus acetate, the glucose uptake does not increase as it does in presence of insulin; (*b*) sheep mammary gland slices metabolizing acetate alone, while unresponsive to insulin, gave evidence of increased fat synthesis in presence of glycerol. Other results, such as the unresponsiveness of sheep udder tissue to insulin in glucose + acetate and the slight inhibitory effect of glycerol in this substrate, are, however, less easy to explain in these terms.

It must be emphasized, however, that this interpretation is at present purely hypothetical. The alternative possibility that the insulin effect is related to the utilization of glucose as a source of

energy for driving the fat synthesis must not be overlooked. If this were the explanation the inertness of ruminant mammary tissue to insulin would be understandable in view of the fact that in ruminants the mammary gland appears to use acetate rather than carbohydrate as an energy source. The whole question clearly needs further investigation.

SUMMARY

1. Insulin added *in vitro* markedly increased $-Q_{O_2}$, acetate and glucose utilization, and especially R.Q. and 'extra CO_2' (carbon dioxide produced in excess of that required for R.Q. = 1) of lactating rat mammary gland slices in glucose plus acetate. This is interpreted as signifying a stimulatory action of insulin on net fat synthesis. Similar but less marked effects were obtained in glucose, but in acetate the tissue was inert to insulin.

2. Rat mammary gland slices were unresponsive to insulin, both in glucose plus acetate and in glucose, at the end of pregnancy (19–20 days) and after 2 days' weaning at days 20–21 of lactation.

3. Lactating rabbit mammary tissue was similarly responsive to insulin in glucose plus acetate.

4. Insulin had no effect on the metabolism of lactating mouse mammary gland in glucose alone. In the absence of insulin, the R.Q. of this tissue was lower in glucose plus acetate than in glucose, but the acid production was somewhat depressed, indicating a slight utilization of acetate. In glucose plus acetate the tissue responded to insulin by moderate increases in $-Q_{O_2}$ and acetate uptake and marked increases in R.Q. and 'extra CO_2'.

5. Udder slices from lactating ewes were inert to insulin both in glucose plus acetate and in acetate.

6. Glycerol exerted a qualitatively similar effect to insulin, though less marked as regards the increases in R.Q. and 'extra CO_2', on the metabolism of lactating rat mammary tissue, except that the glucose uptake was not increased by glycerol. The effects of glycerol and insulin were not additive. Udder slices from lactating ewes responded to glycerol in acetate alone, but in glucose plus acetate glycerol exerted a slight inhibitory effect.

7. It is suggested that the stimulating effect of insulin on fat synthesis by mammary tissue may be due, partly at any rate, to stimulation of the formation of glycerol from glucose. Since mammary tissue can synthesize and secrete glycerides, the supply of glycerol might well be a critical, rate-limiting factor in fat synthesis by this tissue. It is, however, equally possible that the insulin effect is related to the utilization of glucose as an energy source for fat synthesis.

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REFERENCES

Balmain, J. H. & Folley, S. J. (1951). *Biochem. J.* **48**, i.
Balmain, J. H., French, T. H. & Folley, S. J. (1950). *Nature, Lond.*, **165**, 807.
Bloch, K. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 29.
Bloch, K. & Kramer, W. (1948). *J. biol. Chem.* **173**, 811.
Brady, R. O. & Gurin, S. (1950). *J. biol. Chem.* **186**, 461.
Dickens, F. & Šimer, F. (1931). *Biochem. J.* **25**, 973.
Folley, S. J. & French, T. H. (1949*a*). *Biochem. J.* **45**, 117.
Folley, S. J. & French, T. H. (1949*b*). *Biochem. J.* **45**, 270.
Folley, S. J. & French, T. H. (1949*c*). *Biochem. J.* **44**, xlv.
Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.
Folley, S. J. & Young, F. G. (1941). *Lancet*, **1**, 380.
French, T. H. & Popják, G. (1951). *Biochem. J.* **49**, iii.
Gemmill, C. L. & Hamman, L. (1941). *Johns Hopk. Hosp. Bull.* **68**, 50.
Kleiber, M., Smith, A. H. & Tolbert, B. (1950). *XVIIIth Int. Physiol. Congr.* Abstr. p. 304.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Popják, G. & Beeckmans, M.-L. (1949). *Biochem. J.* **44**, xxxvii.
Popják, G. & Beeckmans, M.-L. (1950). *Biochem. J.* **46**, 547.
Popják, G., Folley, S. J. & French, T. H. (1949). *Arch. Biochem.* **23**, 509.
Popják, G., French, T. H. & Folley, S. J. (1950). *Biochem. J.* **46**, xxviii.
Popják, G., French, T. H. & Folley, S. J. (1951). *Biochem. J.* **48**, 411.
Selye, H. (1934). *Amer. J. Physiol.* **107**, 535.
Stadie, W. C., Haugaard, N. & Marsh, J. B. (1951). *J. biol. Chem.* **188**, 167.

The Formation of β-Ketoadipic Acid by Bacterial Fission of Aromatic Rings

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An increasing interest has been shown in recent years in the biological breakdown of aromatic rings, both in the mammalian body and in bacteria. A review of the oxidation of aromatic rings by micro-organisms has been given by Happold (1950) which supplies the historical background to the present investigation. In brief, a study by Evans & Happold (1939) of the metabolism of a vibrio previously isolated from sewage effluent containing spent gas-works liquors (Happold & Key, 1932) demonstrated that phenol was readily metabolized by this organism and could serve as the sole source of carbon and energy, that catechol was formed and disappeared, and that this was followed by the transient appearance of a compound characterized by giving positive Rothera and Gerhardt reactions. It was suspected that this compound might be a keto acid formed after fission of the aromatic ring, but it was not acetoacetic acid as it was much less readily extractable by ether from aqueous solution. The present work was directed towards the isolation and identification of this interesting metabolic intermediate, and some of the results obtained have been briefly reported in a preliminary communication (Kilby, 1948).

MATERIALS

Organism. The vibrio 01 isolated by Happold & Key (1932) from sewage containing gas-works effluent was used. This organism is similar in morphology to *Vibrio cuneata* isolated by Gray & Thornton (1928).

Mineral-salt medium. The mineral-salt medium to which phenol or benzoate was added as a substrate contained $(NH_4)_2SO_4$, 2·5 g.; $Na_2HPO_4.12H_2O$, 1·0 g.; KH_2PO_4, 0·5 g.; metal-salt solution 2·0 ml. and distilled water to 1 l. A stock solution of metal salts was prepared containing $MgSO_4.7H_2O$, 22·5 g.; $FeSO_4.7H_2O$, 2·0 g.; $CuSO_4.5H_2O$, 5·0 ml. of 1% (w/v); $ZnSO_4.7H_2O$, 1·5 ml. of 1% (w/v); $MnCl_2.4H_2O$, 1·5 ml. of 1% (w/v); conc. HCl, 3 ml. and distilled water to 1 l.

β-Ketoadipic acid. Succinic anhydride (Fieser & Martin, 1943) was converted into β-carbethoxypropionyl chloride as described by Reigel & Lilienfeld (1945). Reaction of this with an ethereal suspension of ethyl sodioacetoacetate gave ethyl α-acetyl-β-ketoadipate, b.p. 156°/0·04 mm. The experimental conditions followed and the methods of purification employed were essentially those described by Bardhan (1936) for the preparation of the corresponding methyl ester. A cooled ethereal solution of ethyl α-acetyl-β-ketoadipate was treated with dry NH_3 gas, left overnight, shaken with water, cold dil. HCl and finally with water again, dried with Na_2SO_4, the ether removed, and the product distilled. Ethyl β-ketoadipate was obtained as a colourless oil, b.p. 116–117°/0·015 mm. This ester was hydrolysed by mixing with 9 vol. of conc. HCl and evaporating to dryness over solid KOH in a vacuum desiccator. After two recrystallizations from an ethyl acetate-light petroleum (b.p. 40–60°) mixture, β-ketoadipic acid was obtained as white crystals, m.p. 121° (decomp.), which were very soluble in water. An intense purple colour was given in the Rothera reaction, which could still be detected when a 0·001% solution of the acid was used. On heating the acid alone or in aqueous solution, decarboxylation occurred to give laevulinic acid. Treatment of β-ketoadipic acid with 2:4-dinitrophenylhydrazine gave the 2:4-dinitrophenylhydrazone of laevulinic acid, m.p. 203°.

RESULTS

Isolation of laevulinic acid from a benzoate culture

Mineral-salt medium to which sodium benzoate (1 g./l.) had been added as a substrate was inoculated with the vibrio 01 (previously subcultured in benzoate medium), gently aerated with sterile air and incubated at room temperature. A profuse growth resulted and, after 3 days, the medium, which had remained almost colourless, gave a good Rothera reaction. This culture (3 l.) was then evaporated down to about 50 ml. under reduced pressure and at a temperature not exceeding 37°. A very strong Rothera reaction was given by this concentrate; boiling a sample of it for a few minutes largely destroyed the compound responsible for this test and the reaction became much weaker. Salts which had separated out during the concentration and the bacterial cells were removed by Seitz filtration, the clear solution acidified and the resultant precipitate of unmetabolized benzoic acid removed.

A part of this filtrate was boiled for a few minutes, cooled and extracted continuously overnight with ether. After removal of the solvent from the extract, the residue appeared to be largely benzoic acid, but had a smell reminiscent of laevulinic acid. It was dissolved by shaking with a mixture of water and light petroleum (b.p. 40–60°) and the clear aqueous

layer treated with a solution of 2:4-dinitrophenylhydrazine. A precipitate separated in a few minutes and was recrystallized from methanol forming orange needles, m.p. 203·5°, after drying at 100° *in vacuo*. This product was identified as the 2:4-dinitrophenylhydrazone of laevulinic acid as the melting point was unchanged on mixing with an authentic specimen.

The remainder of the unboiled acidified concentrate was continuously extracted with ether in order to try and obtain the precursor of the laevulinic acid. Although the material responsible for the Rothera reaction disappeared from the aqueous layer, only a small amount was detected in the ethereal extract, presumably due to decomposition in the ether while this was refluxing. The ethereal extract gave the same 2:4-dinitrophenylhydrazone as obtained above.

Isolation of β-ketoadipic acid from a phenolic culture

In the next attempt to isolate without decomposition the compound responsible for the Rothera reaction, which was believed to be acidic in nature, phenol was used as a substrate instead of benzoate in order to avoid a mixture of unchanged benzoic acid and the unknown acid after extraction. The medium (3 l.) containing phenol (1 g.) was inoculated, aerated and incubated at room temperature for 3 days. Good growth resulted, and the slightly yellow medium gave a Rothera reaction. The cells were removed by Seitz filtration, the filtrate adjusted to pH 7·0 and evaporated at 37° to 150 ml. under reduced pressure. The concentrate, after acidification with dilute sulphuric acid, was extracted by hand repeatedly with ether (40 times, using a total of 1·5 l. ether). The ethereal extract was dried with sodium sulphate and the solvent removed under slightly reduced pressure. The residue was dissolved in ethyl acetate and cooled. White crystals separated (30 mg.) which gave an intense permanganate colour with the Rothera reaction. After a further recrystallization from ethyl acetate, crystals were obtained of m.p. 120° (decomp.), unchanged on mixing with authentic synthetic β-ketoadipic acid of m.p. 121° (decomp.).

Breakdown of β-ketoadipic acid by heat

This experiment was carried out to show that the thermal and non-enzymic decomposition of β-ketoadipic acid to laevulinic acid does not give rise to any succinic acid after oxidation of the product with permanganate. A solution of β-ketoadipic acid (26 mg. in 9 ml. 0·1 M-phosphate buffer, pH 7·4) was heated in a boiling-water bath for 30 min., a treatment previously shown to destroy the keto acid. Potassium permanganate (3 ml. of 6 % w/v) and sulphuric acid (1 ml. of 50 % v/v) were added and the mixture was allowed to stand at room temperature for 40 min. It was then extracted for 2·5 hr. with freshly distilled ether in a continuous extractor. Phosphate buffer (2 ml.) was added to the ethereal extract, the ether removed, and the volume made up to 10 ml. with buffer. Succinic acid was estimated manometrically on samples of this solution using the succinic dehydrogenase method (Krebs, 1937). The enzyme preparation used was finely minced and well washed pigeon-breast muscle suspended in five times its volume of phosphate buffer. The succinic acid found in the extract (0·31 mg.) was equivalent to a yield of only 0·03 mol. from 1 mol. of β-ketoadipic acid. (Test runs of the method using 0·5 mg. succinic acid gave 101 % recovery.)

Breakdown of β-ketoadipic acid by the vibrio

The cells from a 4 to 5 day old culture were harvested, washed with distilled water and suspended in about three times their volume of phosphate buffer. The mixtures indicated in Table 1 were incubated at room temperature for 27 hr., in small conical flasks. The cells were removed by centrifugation and washed with 2–3 ml. water, and the combined supernatant and washings heated for 1 hr. in a boiling-water bath. Sodium tungstate (0·25 ml. of 10 % (w/v) solution) was added as a protein precipitant, and permanganate oxidations and succinic acid estimations performed as described previously. Deducting the value of the control found in *C* from the mean of the first two values, the amount of succinic acid found is 14·0 mg. which is equivalent to a yield of 0·95 mol. of succinic acid from 1 mol. of β-ketoadipic acid.

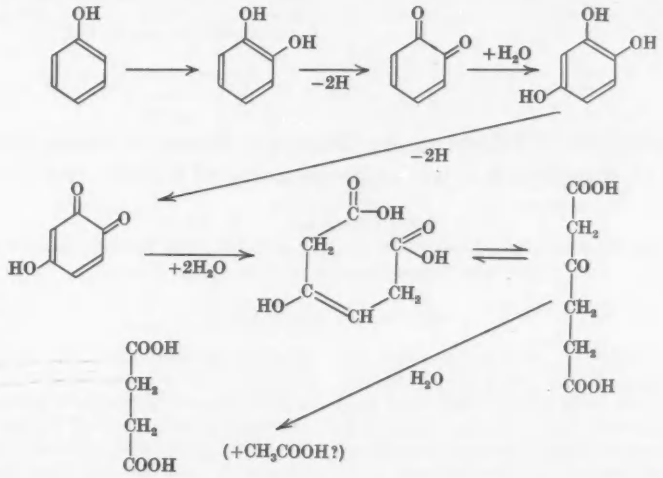
Table 1. *Succinic acid from α-ketoadipic acid in cultures of vibrio* 01

Mixture (see text)	Succinic acid found (mg.)
(*A*) β-Ketoadipic acid, 20 mg. in 1 ml.; 0·1 M-malonic acid, 1 ml.; cell suspension, 1 ml.; buffer, 1 ml.	15·0
(*B*) β-Ketoadipic acid, 20 mg. in 1 ml.; cell suspension, 1 ml.; buffer, 2 ml.	15·4
(*C*) 0·1 M-Malonic acid, 1 ml.; cell suspension, 1 ml.; buffer, 2 ml.	1·17

DISCUSSION

It has been shown by Evans (1947) that the metabolism of benzoate by the vibrio and also by *Pseudomonas fluorescens* proceeds through catechol, and this has been confirmed by Stanier (1950) using his simultaneous adaption technique. It has also been shown that phenol is metabolized by the vibrio through catechol, so that either benzoate or phenol cultures can be used to study changes which occur after the catechol stage as the two metabolites are metabolized along the same pathway.

A scheme for the oxidation of catechol by tyrosinase proposed by Nelson & Dawson (1944) was: catechol → *o*-benzoquinone → 1:2:5-trihydroxybenzene → 5-hydroxy-*o*-benzoquinone → coloured products. The first two of these compounds appear in the medium when phenol is metabolized by the vibrio (Evans, 1947). The similarity between the two systems led the author (Kilby, 1948) to suggest that the metabolism of phenol by the vibrio followed the same pathway as far as 5-hydroxy-*o*-benzoquinone, and that in the presence of tyrosinase this compound was converted into coloured products, while with the vibrio, which is devoid of tyrosinase, a hydrolytic ring fission occurred to give β-ketoadipic acid, which was subsequently broken down to succinic acid and probably acetic acid:



This scheme received support by the isolation soon afterwards of the third postulated intermediate, 1:2:5-trihydroxybenzene, from vibrio cultures growing on catechol (Evans, 1948). Subsequently, Evans and his co-workers have expressed the view (see Happold, 1950) that this compound was not a true bacterial intermediary as it was not oxidized at a sufficient rate to be included in the main metabolic pathway, when oxidation rates were compared using the simultaneous adaption technique (Stanier, 1947).

Since β-ketoadipic acid was first reported as a product of bacterial metabolism (Kilby, 1948), its formation has been confirmed by Evans (1948) and by Stanier, Sleeper, Tsuchida & Macdonald (1950). The latter have shown that specific enzymes capable of oxidizing either catechol or protocatechuic acid to β-ketoadipic acid can be isolated from dried cells adapted to aromatic substrates but not from dried unadapted cells. The rate of oxidation of β-ketoadipic acid by living cells of *Ps. fluorescens* was found by Stanier and his associates always to be much less than that of presumed aromatic precursors, perhaps because the highly polar acid penetrates slowly into the cell.

It appears, therefore, that β-ketoadipic acid has been established as an intermediate in the bacterial metabolism of some aromatic compounds, but the nature of its immediate precursors is still an open question. The almost quantitative conversion of β-ketoadipic acid into succinic acid which was observed suggests that a hydrolytic split of the keto acid into succinic and acetic acids is the next stage in the metabolic pathway. This would be analogous to the fission of acetoacetic acid into acetic acid observed by Lehninger (1942) working with *Escherichia coli*. Whether the fission of the keto acid is followed by oxidation appears to depend on the experimental conditions; when left at room temperature without shaking, very little of the succinic acid formed was metabolized, and addition of malonate had little effect. Shaking in Warburg flasks at 37° gave oxygen uptakes which varied from about 3 to 11·3 atoms of oxygen/molecule of keto acid depending on the sample of cells used (for complete oxidation, 11 atoms are required).

The metabolism of aromatic compounds in the mammal may follow a somewhat similar pathway. Phenylalanine and tyrosine are metabolized by rat-liver slices through homogentisic acid, and Lerner (1949) believes that ring fission occurs at one point to give an 8-carbon dicarboxylic acid from which acetoacetate and malic acid are subsequently formed.

SUMMARY

1. β-Ketoadipic acid has been shown to arise by ring fission in the metabolism of phenol and benzoic acid by a vibrio species.

2. The keto acid is subsequently degraded by the vibrio to succinic acid.

The author wishes to express his thanks to Prof. F. C. Happold for suggesting the problem and for his interest and help during the work.

REFERENCES

Bardhan, J. C. (1936). *J. chem. Soc.* p. 1848.
Evans, W. C. (1947). *Biochem. J.* **41**, 373.
Evans, W. C. (1948). Private communication.
Evans, W. C. & Happold, F. C. (1939). *J. Soc. chem. Ind., Lond.*, **58**, 55.
Fieser, L. F. & Martin, E. L. (1943). *Organic Synth. Coll.* **2**, 560.
Gray, P. H. H. & Thornton, H. G. (1928). *Zbl. Bakt.* (II), **73**, 74.
Happold, F. C. (1950). *Biochem. Soc. Symp.* **5**, 85.
Happold, F. C. & Key, A. (1932). *J. Hyg., Camb.*, **32**, 573.
Kilby, B. A. (1948). *Biochem. J.* **43**, v.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Lehninger, A. L. (1942). *J. biol. Chem.* **143**, 147.
Lerner, A. B. (1949). *J. biol. Chem.* **181**, 281.
Nelson, J. M. & Dawson, C. R. (1944). *Advanc. Enzymol.* **4**, 99.
Reigel, B. & Lilienfeld, W. M. (1945). *J. Amer. chem. Soc.* **67**, 1273.
Stanier, R. Y. (1947). *J. Bact.* **54**, 339.
Stanier, R. Y. (1950). *Bact. Rev.* **14**, 179.
Stanier, R. Y., Sleeper, B. P., Tsuchida, M. & Macdonald, D. L. (1950). *J. Bact.* **59**, 137.

Comparison of Haem *a*, the Dichroic Haem of Heart Muscle, and of Porphyrin *a* with Compounds of Known Structure*

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In his classical studies on the photochemical absorption spectrum of the respiratory ferment, Warburg (for review see Warburg, 1949) concluded that its prosthetic group is a haem with a formyl side chain, related to chlorocruorohaem ('Spirographis' haem) or to phaeohaem b_6 (iron complex of compound, formula III). The absorption spectra of the cytochromes *a*, a_1 and a_3 (the last named probably identical with cytochrome oxidase and the respiratory ferment) suggest that these are all derived from the same type of haem and possibly the same haem (cf. Lemberg & Legge, 1949). Attempts to isolate this haem from muscle have led to somewhat controversial results. It has been possible to isolate such a haem from heart muscle (Negelein, 1933; Roche & Bénévent, 1936), but there has been no complete agreement as to its properties. The porphyrin ('cryptoporphyrin') isolated by Negelein (1932*a*) from pigeon-breast muscle was later considered an artifact by this same author (1932*b*); this may well be so, but we have not been able to confirm Negelein's opinion that it arose through the action of light on protoporphyrin in acid solution during the isolation (cf. also Rawlinson & Hale, 1949). The divergences were probably due to the difficulties of isolation and to the lability of haem *a*, and not to the existence of a variety of haems *a* derived from different cytochromes, although such a possibility has not yet been definitely excluded.

At the time of Warburg's investigations only a small number of porphyrin derivatives with carbonyl groups in the side chains was known, but it has been greatly increased since, particularly through the work of Fischer. It appeared necessary to compare their properties and the properties of their haem compounds systematically with those of porphyrin *a* and haem *a*. For this it was necessary to reinvestigate the properties of the latter compounds. This study had just begun when, through the kindness of Dr W. A. Rawlinson, we were informed of the isolation of haem *a* and porphyrin *a* from ox-heart muscle and from *Corynebacterium diphtheriae* by an improved method (Rawlinson & Hale, 1949). We have, therefore, concentrated mainly on the comparison of these products with porphyrins and haems of known structure containing carbonyl groups in conjugation with the nucleus in one or two pyrrole rings. The three schools working on haem *a* (at the Department of Chemical Pathology, University

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College Hospital Medical School, London, at the Department of Biochemistry, University of Melbourne, and the Institute of Medical Research, Royal North Shore Hospital of Sydney), have collaborated for some time and presented three conjoint papers to the First International Congress of Biochemistry at Cambridge (see Rimington, Hale, Rawlinson, Lemberg & Falk, 1949). Close contact is maintained, but to minimize publication difficulties each group reports separately the work it has performed.

The properties of haemochromogen *a* support the previous assumption (Warburg, 1949) of an aldehyde (formyl) side chain. Stern and his co-workers (Stern & Wenderlein, 1934, 1935*a–c*, 1936; Stern & Molvig, 1936), have found correlations between the nature and orientation of carbonyl side chains on porphyrins, on the one hand, and the type of neutral porphyrin spectrum on the other. Porphyrins without such groups have, as a rule, an aetio-type spectrum in which the intensity of the bands increases stepwise from band I (that at longest wavelengths) to band IV (at shortest wavelengths). The order of intensities in this type of spectrum is conventionally stated as IV, III, II, I (Fig. 1*a*). The introduction of a single carbonyl group conjugated with the nucleus produces a rhodo-type spectrum (order of intensities: III, IV, II, I; cf. Fig. 1*b*). The conversion of the aldehyde or ketone into an oxime or hydrazone abolishes or greatly decreases the 'rhodofying effect' of the side chain. Introduction of a second carbonyl group on a pyrrole ring vicinal to the one substituted by the first, abolishes the effect of the first and leads to a spectrum of aetio type. Introduction of the second carbonyl group on a pyrrole ring opposite to the one substituted by the first, leading for example to carbonyl substitution on rings I and III, enhances the effect of the first with appearance of a spectrum similar to that of oxorhodoporphyrin (XXI), which we therefore call oxorhodo type (order of intensities III, II, IV, I; cf. Fig. 1*c*).

These correlations have been found to hold good in all instances examined, even where earlier data indicated exceptions, and together with other experimental data they allow certain conclusions about the structure of porphyrin *a* to be drawn. Such an approach to the problem is particularly appropriate in view of the great difficulty in obtaining haem *a* in a pure state in amounts sufficient for chemical studies. More evidence will, however, be required before the number of possible structures is limited to such a degree that an attempt at synthesis can be made with hope of success. One way in which we are seeking more evidence is through a systematic study of the infrared spectra.

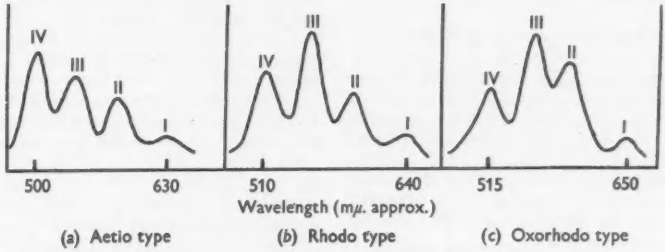


Fig. 1. Types of spectra of porphyrins in the visual region, in neutral solvents.

EXPERIMENTAL

Methods

Melting points were determined with a Gallenkamp electric hot-stage micro-melting point apparatus, and were corrected.

Absorption spectra were measured with a Hilger-Nutting visual spectrophotometer, a Beckman photoelectric spectrophotometer or a Beck-Hartridge reversion spectroscope, as indicated in the tables.

Dioxan was purified by the method of Eigenberger (1931). A solution of protoporphyrin ester in this dioxan did not change in spectroscopic properties after standing immersed in a boiling-water bath for 2 hr. in subdued light. This is a sensitive test; in the presence of traces of peroxides protoporphyrin is quickly oxidized, a strong absorption band appearing at 660 mμ. after a few minutes.

Hydrogen peroxide-ether. H_2O_2 (30%) was extracted with ether for several days in a liquid-liquid extractor, and the ethereal H_2O_2 solution dried over anhydrous Na_2SO_4. For titration 1 ml. was diluted to 100 ml. with distilled water; 10 ml. of this solution required 2·4 ml. 0·1 N-$KMnO_4$.

Hydrochloric acid concentrations. Because the familiar HCl number (Willstätter number), widely used in the purification of porphyrins, is stated in terms of % (w/v) HCl, this form is used instead of normality (1% HCl ≡ 0·274 N).

Haemins or their esters were prepared from the corresponding porphyrins or esters by the ferrous acetate-acetic acid method of Warburg & Negelein (1932*a*), except chlorocruorohaemin and haemin *a*, which were isolated from natural materials.

Oximes of porphyrins containing carbonyl groups were prepared as follows. The porphyrin ester was dissolved in pyridine, and to the solution, refluxing gently on a sand bath, an excess of a mixture of equivalent amounts of solid hydroxylamine hydrochloride and Na_2CO_3 was added. Refluxing was continued for 15 min., the solution cooled and the products taken into ether. After washing the ether solution free from pyridine with water, it was dried over anhydrous Na_2SO_4. When sufficient material was available the oximes were crystallized, as described below.

Substances used in this investigation

Monoacetyldeuteroporphyrin IX dimethyl ester. This was a small sample from the collection of the late Prof. H. Fischer, kindly made available by Prof. S. Goldschmidt. Its mode of preparation is not known to us, but its m.p. (243·5°) is close to that of the 4-acetyl isomer described by Fischer & Wecker (1942).

Oxime. A sample sufficient only for spectroscopy was made.

2:4-*Diacetyldeuteroporphyrin IX dimethyl ester* (*formula XI, Table* 2). Deuterohaemin, prepared from haemin by the resorcinol fusion method of Schumm (1928) was acetylated with acetic anhydride in the presence of $SnCl_4$ at 4° (Fischer & Zeile, 1929). From the crude diacetyldeuterohaemin, the porphyrin ester was prepared by the method of Grinstein (1947). After separation from by-products by ether-HCl fractionation, the ester was crystallized and twice recrystallized from $CHCl_3$-acetone; m.p. 236°.

Dioxime. About 5 mg. of the porphyrin ester was converted to the dioxime.

Monoformyldeuteroporphyrin IX dimethyl ester. Deuterohaemin, prepared as above, was converted by the method of Grinstein (1947) to deuteroporphyrin ester. This was saponified and purified by ether-HCl fractionation; after re-esterification and three crystallizations from $CHCl_3$-methanol its m.p. was 224°. From this deuteroporphyrin ester, deuterohaemin ester was prepared by the ferrous acetate method. The deuterohaemin ester was formylated by the dichloromethyl-ethyl-ether method of Fischer & Wecker (1942) (cf. also Fischer & Beer, 1936), and the crude formyldeuterohaemin converted to porphyrin by stirring for 10 min. in conc. H_2SO_4. The acid solution was thrown into crushed ice, and after further dilution the porphyrin was taken into ether. After washing the ether solution until neutral, the crude formyldeuteroporphyrin was extracted into 7% (w/v) HCl, precipitated by sodium acetate, collected at the pump and dried. Removal of iron from the crude formyldeuterohaemin by conc. H_2SO_4, by the ferrous acetate method (Warburg & Negelein, 1932*a*) and by the method of Grinstein (1947), gave products with similar absorption in the visible region. The crude monoformyldeuteroporphyrin was now esterfied with methanol saturated with dry HCl, and the ester crystallized from $CHCl_3$-methanol. From this material (m.p. 217°) extraction with hot methanol removed a small fraction of m.p. 221–224° and extraction of the remainder by $CHCl_3$, followed by two crystallizations from $CHCl_3$-methanol, led to a product of m.p. 231°. This presumably corresponds to the product of Fischer & Wecker (1942), of m.p. 232°, which they stated to be a mixture of the 2- and 4-formyl isomers. Neither of their two methods for the isolation of the 4-isomer (m.p. 260°) was successful in our hands. For our purposes the separation was not of great importance.

Oxime. From the porphyrin ester of m.p. 231° the oxime was prepared as previously described, and after recrystallization from $CHCl_3$-methanol had m.p. 222–223°. Fischer & Beer (1936) described an oxime of m.p. 226°; Fischer & Wecker (1942) described no simple derivatives of their porphyrin of m.p. 260°.

2:4-*Diformyldeuteroporphyrin IX dimethyl ester* (*formula X*). This was prepared from pure protoporphyrin dimethyl ester (m.p. 230°) by OsO_4-H_2O_2 oxidation, by a modification of the method of Fischer & Deilmann (1944). Protoporphyrin ester (100 mg.) was dissolved in 40 ml. dioxan by heating on a steam bath. To this, 2 ml. of a solution of 1 g. OsO_4 in 100 ml. dry ether and 11 ml. H_2O_2-ether (cf. p. 675) were now added, and the mixture refluxed vigorously for 5 min., the protoporphyrin band at 630 mμ. now being no longer visible by the hand spectroscope. Longer heating decreases the yield. In a series of experiments the yields after 5, 15 and 90 min. refluxing were 12, 7·2 and 2·7%, respectively. When, instead of refluxing, the reaction mixture was allowed to stand for 30 min. at room temperature (19°) a yield of 8·7% was obtained. Unreacted protoporphyrin makes working up much more difficult, and the reaction must be stopped when the hand spectroscope shows that the protoporphyrin has just disappeared. This was done by pouring into 2 vol. water, from which the products were extracted with $CHCl_3$. The $CHCl_3$ solution was washed ten times with water, dried over anhydrous Na_2SO_4, and passed through an alumina column (British Drug Houses Ltd. 'For chromatographic adsorption analysis', or Savory & Moore 'nach Brockmann'). The diformyldeuteroporphyrin ester passed through the column faster than the various by-products, which mostly remained as a strongly adsorbed, dark-coloured band at the top of the column; on development with $CHCl_3$ most of the diformyl compound ran through before a small green-yellow band which followed it. The latter contains a porphyrin with a rhodo-type spectrum, and in addition a substance with a strong band at 660 mμ. The $CHCl_3$ solution of the diformyl compound was chromatographed again in the same way through fresh alumina, and, examined by the hand spectroscope, then appeared to have a pure diformyldeuteroporphyrin spectrum. The $CHCl_3$ solution was then concentrated *in vacuo* to about 2 ml. and, on standing for a short time, the ester crystallized. On recrystallization from $CHCl_3$-acetone, 12 mg. of material of m.p. 280° (decomp.) were obtained. The absorption spectrum in $CHCl_3$ was identical with that of Fischer & Deilmann's (1944) product as far as could be determined from their small published curve (cf. Table 1).

Dioxime. Prepared by the method of Fischer & Deilmann (1944). On crystallization from $CHCl_3$ its m.p. was 231–232°, unchanged by recrystallization.

Copper complex. This was prepared by the reaction of a solution of the porphyrin ester in glacial acetic acid with an acetic acid solution of copper acetate. The copper complex crystallized from the reaction mixture and was recrystallized from $CHCl_3$-methanol; m.p. 290°. Absorption spectrum in the reversion spectroscope: in $CHCl_3$, I, 597 mμ.; II, 553 mμ. Order of intensities: I, II. In pyridine-ether: I, 594·5 mμ.; II, 554·6 mμ. Order of intensities: I, II.

'*Oxorhodoporphyrin*' (*2-acetyl-4-ethyl-1:3:5:8-tetramethylporphin-6-carboxylic acid-7-propionic acid*) *dimethyl ester* (*formula XXI*). This was prepared by the method of Fischer & Krauss (1936). The porphyrin obtained after long and tedious ether-HCl fractionations had a spectrum similar to that found by Stern & Wenderlein (1935*a*). It was esterified

by methanol HCl, and the ester, crystallized from pyridine, had m.p. 280°. The yield was minute.

Oxime. This was prepared, in amounts sufficient for spectroscopic study only, by the method described above.

Porphyrin obtained by the action of daylight on protoporphyrin in HCl *solution.* Pure protoporphyrin (100 mg.) was dissolved in 200 ml. 10% (w/v) HCl, the solution transferred to Roux flasks so that the liquid layers were about 1 cm. deep, and the flasks allowed to stand on a shelf exposed to sunlight for 13 days. The solution was filtered and the porphyrins in the filtrate taken into ether by dilution with water. The ether was extracted exhaustively with 2% (w/v) HCl to remove unchanged protoporphyrin. The colour of the remaining ether solution was green with a pink tinge. Fractionation of this solution with increasing concentrations of HCl showed that the ether contained only one porphyrin, which was extracted quantitatively by 10% (w/v) HCl as a bright-green solution. The absorption curves in HCl and in ether gave no indication that more than one substance was present.

Spectrophotometric data for the porphyrin in ether are shown in Table 1, and for the pyridine haemochromogen prepared from it in Table 3. The porphyrin in 10% (w/v) HCl had the following bands (Hilger-Nutting visual spectrophotometer): I, 610; II, 565 mμ. Ratio I:II, 0·515.

Chlorocruoro ('Spirographis') porphyrin dimethyl ester (IX). A sample of chlorocruorohaemin prepared by Dr J. H. Hale from *Sabella pavonina* and *Branchiomma vesiculosum* was converted to the porphyrin by the ferrous acetate method of Warburg & Negelein (1932*a*). An ether solution of the product was extracted with 1·5% (w/v) HCl to remove any traces of protoporphyrin. The chlorocruoroporphyrin was esterfied with HCl-saturated methanol and crystallized from $CHCl_3$-ether; m.p. 270°.

Oxime. Prepared as described above. Unfortunately we were unable to obtain sufficient of this material for an analysis. No melting point for the analytically pure oxime ester has been found in the literature.

Chlorocruorohaem was extracted from the marine worm *Sabella starte indica* (formerly *Spirographis australiense*) collected on rock platforms on the coast about Sydney. Worms (120 g. of fresh worms) were minced and extracted with water. To the 500 ml. of aqueous extract so obtained, 2 l. of ice-cold acetone containing 10 ml. of 2N-HCl were added. The mixture stood at 0° for 2 hr., and then the precipitate of protein and other insoluble material was filtered off. The acetone was removed from the clear brown filtrate under vacuum, and the crude chlorocruorohaemin which separated from the remaining aqueous HCl was recovered at the centrifuge, dried *in vacuo* over $CaCl_2$ and crystallized from glacial acetic acid. Yield, 0·5 mg. No attempt was made to remove traces of protohaemin, which, although no doubt extracted from the body tissues of the worms by this procedure, could not be detected spectroscopically.

Phaeoporphyrin a_5 dimethyl ester (formula II) was a sample from the collection of Fischer. Amounts of 10 mg. each were converted into the oxime and into crystalline phaeohaemin a_5.

Phylloerythrin methyl ester (formula IV) was a pure sample prepared from sheep bile by Prof. C. Rimington.

Haemin a. This was isolated in minute quantities from ox hearts by the method of Rawlinson & Hale (1949). The haemochromogen had spectroscopic properties identical with those described by these authors.

RESULTS

Porphyrins

Spectrophotometric data concerning the porphyrins and their oximes are shown in Tables 1 and 2. Among the compounds of known structure, the spectra all conform with Stern's correlations. The spectra of phaeoporphyrin a_5 (II) and phylloerythrin (IV), which contain the isocyclic ring, and of their oximes, are discussed below. All the other oximes were aetio type except that of oxorhodoporphyrin (XXI), which retains the 'rhodofying' carbonyl group. Porphyrin *a* and its oxime have spectra of the same types as oxorhodoporphyrin and its oxime, and the possible significance of this is discussed below.

As shown in Table 1, spectra in chloroform, in comparison with other organic solvents, tend towards oxorhodo type, i.e. bands II and III are intensified in relation to band IV and the absorption maxima are moved to slightly longer wavelengths. This effect of chloroform has been observed consistently in many porphyrins besides those shown here.

Haemins

Haemochromogen spectra of the compounds studied are shown in Table 3. We have confirmed the finding of Fischer & Krauss (1936) that oxorhodohaemochromogen has only a single absorption band in the visible region, and have found that the haemochromogen of phylloerythrin haemin dimethyl ester has only a very faint second band in the visible region (Table 3). In the same table the spectra are shown of the haemochromogens obtained after reaction with hydroxylamine or with hydrazine.

Reaction with hydroxylamine

To a solution of the haemin in 0·5 ml. pyridine, 1·5 ml. of distilled water were added, followed by about 3 mg. of a mixture of equivalent amounts of solid hydroxylamine hydrochloride and sodium carbonate. Solution of the solids was effected by gentle rotation, and the solution allowed to stand for 30 min. at about 20°. A pinch of sodium dithionite was added for full reduction before measuring the haemochromogen absorption bands of the product.

Under these conditions the formyl compounds and haemochromogen *a* reacted with hydroxylamine with a considerable shift of absorption bands towards shorter wavelengths; the acetyl compounds and phaeohaemin a_5 and phylloerythrin haemin gave little or no shift (Table 3).

Reaction with hydrazine

This reaction was carried out under the same conditions as the hydroxylamine reaction, using hydrazine sulphate instead of hydroxylamine

Table 1. *Absorption spectra of porphyrins and porphyrin oximes*

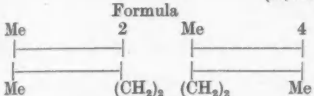
(Δ Band I indicates the magnitude of the shift to shorter wavelengths of this band on oxime formation. *A*, aetio type; *R*, rhodo type; *O*, oxorhodo type. Negelein's 'cryptoporphyrin', Rawlinson & Hale's porphyrin *a* and our product from the action of light on protoporphyrin were the free porphyrins; the remainder were methyl esters.)

Compound	Formula	Solvent	Absorption maxima (mμ.) and ratios of intensities (in parentheses) I		II		III		IV		Spectral type	Δ Band I (mμ.)
Monoformyldeuteroporphyrin	(VIII)	Dioxan*	640	(0·223)	578·5	(0·850)	551	(1·430)	512	(1·0)	*R*	—
		Chloroform*	641	(0·175)	580	(1·037)	555	(1·790)	515	(1·0)	*R–O*	—
Monoformyldeuteroporphyrin oxime		Dioxan*	630	(0·225)	575	(0·558)	540	(0·948)	504	(1·0)	*A*	10
		Dioxan†	628	(0·225)	573	(0·507)	539	(0·944)	503	(1·0)	*A*	12
		Chloroform*	630	(0·213)	574	(0·598)	543	(1·010)	506	(1·0)	*R–A*	11
2:4:Diformyldeuteroporphyrin	(X)	Dioxan*	650	(0·184)	593	(0·458)	559	(0·585)	523	(1·0)	*A*	—
		Chloroform‡	651	(0·228)	595	(0·490)	562	(0·600)	525	(1·0)	*A*	—
		?§	648	(0·267)	594	(0·466)	561	(0·580)	525	(1·0)	*A*	—
2:4-Diformyldeuteroporphyrin dioxime		Dioxan*	639	(0·309)	583	(0·485)	546	(0·785)	512	(1·0)	*A*	11
		Chloroform‡	640	(0·285)	582	(0·485)	550	(0·820)	514	(1·0)	*A*	11
Monoacetyldeuteroporphyrin		Chloroform‡	634	(0·193)	578	(0·735)	548	(1·145)	511	(1·0)	*R*	—
Monoacetyldeuteroporphyrin oxime		Chloroform‡	622	(0·237)	570	(0·500)	536	(0·732)	502	(1·0)	*A*	12
2:4-Diacetyldeuteroporphyrin	(XI)	Dioxan*	639	(0·246)	588	(0·425)	552	(0·525)	516	(1·0)	*A*	—
		Chloroform‡	640	(0·258)	588	(0·460)	552	(0·545)	518	(1·0)	*A*	—
2:4-Diacetyldeuteroporphyrin dioxime		Dioxan*	625	(0·220)	576	(0·409)	537	(0·588)	503	(1·0)	*A*	14
		Chloroform‡	626	(0·253)	573	(0·484)	538	(0·632)	506	(1·0)	*A*	14
Oxorhodoporphyrin	(XXI)	Dioxan‖	637	(0·270)	583	(1·280)	557·5	(2·395)	515	(1·0)	*O*	—
Oxorhodoporphyrin oxime		Dioxan†	631	(0·089)	575	(0·892)	548	(1·747)	509	(1·0)	*R*	6
Protoporphyrin irradiation product		Ether‡	640	(0·161)	583	(0·515)	548	(0·807)	511	(1·0)	*A*	—
'Cryptoporphyrin'		?¶	648–639		591–579·5		559–549		520–505		*R*	—
Phylloerythrin	(IV)	Dioxan*	637·5	(0·224)	583·5	(0·984)	559	(1·469)	519	(1·0)	*O–R*	—
		Dioxan‖	634	(0·218)	581·5	(1·000)	557·5	(1·517)	517·5	(1·0)	*O–R*	—
Phylloerythrin oxime		Dioxan*	628·5	(0·145)	574	(0·675)	549	(0·993)	512	(1·0)	*R–A*	9
Phaeoporphyrin a_5	(II)	Dioxan‖	634	(0·202)	583	(1·343)	562	(1·710)	521	(1·0)	*O*	—
		Chloroform‡	639	(0·310)	588	(1·550)	568	(1·640)	525	(1·0)	*O*	—
Phaeoporphyrin a_5 oxime		Dioxan†	625	(0·150)	573	(0·833)	550	(1·117)	512	(1·0)	*R*	9
		Chloroform‡	627	(0·170)	576	(1·100)	556	(1·370)	517	(1·0)	*O*	12
Porphyrin *a*		Dioxan*	648	(0·400)	582	(1·040)	557	(1·640)	515	(1·0)	*O*	—
		Pyridine**	647	(0·301)	582	(1·097)	559	(1·512)	516	(1·0)	*O*	—
Porphyrin *a* oxime		Pyridine**	643	(0·229)	578	(0·724)	551	(1·233)	512	(1·0)	*R*	4
Porphyrin *a* ester		Chloroform‡	645	(0·275)	585	(1·310)	562	(1·780)	518·5	(1·0)	*O*	—

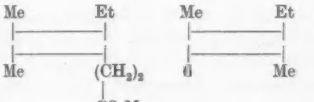
* Determined by the Beckman photoelectric spectrophotometer.
† From Stern & Wenderlein (1936).
‡ Determined by Hilger-Nutting visual spectrophotometer.
§ Calculated from small curve published by Fischer & Deilmann (1944).
‖ From Stern & Wenderlein (1935*a*).
¶ From Negelein (1932*a*).
** Calculated from Rawlinson & Hale (1949).

Table 2. *The effect of certain substituents on the wavelength of maximal absorption, and on the type of spectrum in the visual region, of porphyrin methyl esters and their oximes in dioxan solution*

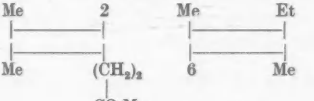
(*A*, aetio type; *R*, rhodo type; *O*, oxorhodo type.)



Formula	Substituents at positions 2	Substituents at positions 4	Band I max. (mμ.)	Spectral type (cf. Fig. 1)
V	—H	—H	618*†	*A*
VI	—C_2H_5	—C_2H_5	620*†	*A*
VII	—CH:CH_2	—CH:CH_2	630*†	*A*
VIII	—H	—CHO	640*	*R*
	—H	—C:N.OH	630* 628†	*A*
IX	—CHO	—CH:CH_2	641* 639†	*R*
	—C:N.OH	—CH:CH_2	635·5*	*A*
X	—CHO	—CHO	650*	*A*
	—C:N.OH	—C:N.OH	639*	*A*
XI	—$CO.CH_3$	—$CO.CH_3$	639*	*A*
	—C(:N.OH).CH_3	—C(:N.OH).CH_3	625*	*A*

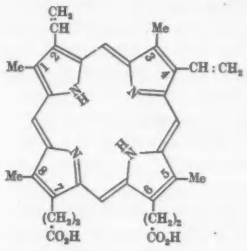


Formula	Substituents at position 6	Band I max. (mμ.)	Spectral type (cf. Fig. 1)
XII	—H	620†	*A*
XIII	—CH:CH_2	624†	*A*
XIV	—CHO	635·5†	*R*
XV	—$CO.CH_3$	632†	*R*
XVI	—$CO.C_6H_5$	627†	*R*
XVII	—$COOCH_3$	632†	*R*
XVIII	—CH:CH.$COOCH_3$	624·5†	*R*

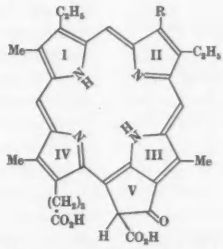


Formula	Substituents at positions 2	Substituents at positions 6	Band I max. (mμ.)	Spectral type (cf. Fig. 1)
XIX	—C_2H_5	—$COOCH_3$	632†	*R*
XX	—CH:CH_2	—$COOCH_3$	638·5†	*R-O*
XXI	—$CO.CH_3$	—$COOCH_3$	637†	*O*
	—C(:N.OH)CH_3	—$COOCH_3$	631†	*R*
XXII	—$COCH_3$	—$COCH_3$	637†	*O*
XXIII	—CHO	—$COOCH_3$	Unknown	Unknown

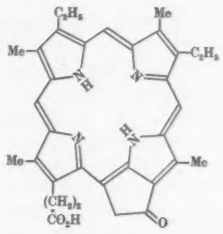
* Measured for this study (cf. Table 1). † From the data of Stern *et al.* (1934–6).



I Protoporphyrin IX.



II R = —CH_3. Phaeoporphyrin a_5.
III R = —CHO. Phaeoporphyrin b_5.



IV Phylloerythrin.

Table 3. *Absorption spectra of haemochromogens, before and after reaction with hydrazine and with hydroxylamine*

(All measurements were made by the Beck-Hartridge reversion spectroscope. Δ Band I indicates the magnitude of the shift to shorter wavelengths of this band after reaction.)

		Haemochromogen		Haemochromogen after hydroxylamine reaction			Haemochromogen after hydrazine reaction		
Haemin of	Formula	Band I (mμ.)	Band II (mμ.)	I	II	Δ Band I (mμ.)	I	II	Δ Band I (mμ.)
Monoformyldeuteroporphyrin	—	578	548	560	530	18	560	530	18
2:4-Diformyldeuteroporphyrin	X	584·3	549·7	563	533	21	558	527	24
Monoacetyldeuteroporphyrin	—	571	530	569·5	530	1·5	571	530	Nil
2:4-Diacetyldeuteroporphyrin	XI	575·2	539·7	573	530·1	2·2	575·2	539·7	Nil
Chlorocruoroporphyrin	IX	583·1	545·1	561·4	529·5	22	553	523	30
Oxorhodoporphyrin	XXI	582	—	582	—	Nil	582	—	Nil
Haemin *a*	—	587	—	570	533	17	569	530	18
Haemin from protoporphyrin irradiation product	—	576	530	—	—	—	—	—	—
'Cryptohaemin'	—	582	531	—	—	—	—	—	—
Phaeohaemin a_5	—	587	(555·5)	587	(555)	Nil	587	(555)	Nil
Phylloerythrin haemin	—	584	(538)	584	(538)	Nil	584	(538)	Nil

hydrochloride. In these experiments the formyl compounds and haemochromogen *a* again reacted with a shift of absorption bands to shorter wavelengths, but the acetyl compounds, and phaeohaemin *a* and phylloerythrin did not (Table 3).

Warburg, Negelein & Haas (1930) had noticed a similar reaction with chlorocruorohaemin; they reported that the (pyridine hydrazine) haemochromogen was unstable, the 585 mμ. band wandering to 560 mμ. 'under conditions we do not yet understand'.

A similar shift in the absorption bands of haemochromogen *a* caused by the action of cysteine (Rawlinson & Hale, 1949) had already indicated the presence of a formyl group in this material. Cysteine causes a similar shift in the absorption bands of phaeohaemin a_5 in weakly alkaline solution at 75° (cf. also Fischer & Mittermair, 1941). In 25% pyridine solution, however, cysteine does not shift the absorption bands of phaeohaemochromogen a_5 at room temperature.

The spectroscopic changes in our hydroxylamine and hydrazine reactions are thus almost certainly not due to the reaction of a keto group, but confirm the presence of a formyl group (cf. Rawlinson & Hale, 1949).

Porphyrin from action of daylight on protoporphyrin in hydrochloric acid solution

Negelein (1932*a*) isolated from pigeon-breast muscle a porphyrin which he called 'cryptoporphyrin' (cf. Table 1). A little later (1932*b*) he reported, giving no details of either experimental method or products, that by the action of light on protoporphyrin in hydrochloric acid solution a porphyrin identical with 'cryptoporphyrin' was obtained. He concluded that 'cryptoporphyrin' was an artifact arising from protoporphyrin during the isolation from the muscle. We therefore made an attempt to repeat Negelein's irradiation experiment (see Experimental section), choosing daylight in the absence of more specific information. Our product was neither in band position nor in spectroscopic type identical with Negelein's (1932*a*) 'cryptoporphyrin' (Table 1), nor were the corresponding haemochromogens identical (Table 3). Though we may have obtained a different product, in our irradiation experiment, from Negelein's (1932*b*) product, the band positions of our 'irradiated protoporphyrin' lie close enough to those of Negelein's (1932*a*) 'cryptoporphyrin' to make it conceivable that by direct vision spectroscopy the substances might be thought to be identical.

Chlorocruoroporphyrin ('Spirographis' porphyrin)

The structure (IX, Table 2) for this porphyrin rests on very strong evidence (Fischer & Seemann, 1936; Fischer & Wecker, 1942; Fischer & Deilmann, 1944). The porphyrin itself has been the subject of some careful spectrophotometric studies (Fischer & Seemann, 1936; Stern & Molvig, 1936), but the oxime and other derivatives have been studied solely by direct vision spectroscopy, from which only unreliable estimates of relative intensities can be made. Reports based on such observations have not been consistent with the correlations of Stern and coworkers. Thus Fischer & Seemann (1936) reported no movement of band I (in pyridine) and a rhodo-type spectrum after oxime formation. Warburg & Negelein (1932*a*, *b*), however, did observe a shift of the absorption bands to shorter wavelengths on oxime formation.

By spectrophotometric measurements (Table 4) we have found in the oxime an aetio-type spectrum and a marked shift to shorter wavelengths, in accordance with Stern's correlations.

Table 4. *Absorption spectra of chlorocruoroporphyrin ('Spirographis' porphyrin) ester and its oxime*

Compound	Solvent	Absorption maxima (mμ.) and ratios of intensities (in parentheses) I	II	III	IV	Δ Band I (mμ.)
Porphyrin ester	Chloroform	644 (0·209)	584 (0·917)	558·5 (1·400)	518·5 (1·0)	—
	Dioxan	641 (0·213)	583 (0·728)	554 (1·210)	515 (1·0)	—
	Dioxan*	639 (0·223)	581 (0·770)	553 (1·204)	514 (1·0)	—
Porphyrin ester oxime	Chloroform	634 (0·270)	579 (0·558)	546 (0·928)	511 (1·0)	10
	Dioxan	635·5 (0·277)	580 (0·537)	543 (0·874)	508 (1·0)	5·5
	Ether	636 (0·294)	580 (0·469)	541 (0·870)	505 (1·0)	—

* From Stern & Molvig (1936); the remainder of the measurements were made by the Beckman spectrophotometer.

DISCUSSION

All side chains having double bonds in conjugation with the porphyrin nucleus shift the absorption spectrum towards longer wavelengths, the shift being particularly noticeable in band I. As shown in Table 2, aldehyde and acrylic acid groups have a stronger effect in this direction than keto groups, which in turn are stronger than vinyl groups.

Further, a $\rangle C{=}O$ group in an aldehyde, a ketone or a carboxylic acid side chain, causes enhancement of the absorption of band III relative to that of band IV with the appearance of a rhodo-type spectrum (cf. Fig. 1*b*). The acrylic acid side chain also causes a rhodo-type spectrum to appear; unfortunately only one reliable example for this effect of the acrylic acid group is available (XVIII). Such substituents which, singly, cause the appearance of a rhodo-type spectrum have been called (Lemberg, Falk, Rimington, Hale & Rawlinson, 1949) 'rhodofying' groups. The vinyl group, and aldoxime and ketoxime groups are not rhodofying. Thus (XIII) has an aetio-type spectrum and while (VIII), (IX) and (XIV–XVIII) have rhodo-type spectra, the oximes of (VIII) and (IX) have spectra of aetio type. In addition, the large shift of absorption bands to longer wavelengths due to carbonyl groups is partly reversed on oxime formation, the oxime group in its effect both on wavelength of absorption and on type of spectrum thus resembling a vinyl group.

Two rhodofying groups on vicinal pyrrole rings, e.g. (III), (X) and (XI), abolish each other's rhodofying effect, and the aetio-type spectrum is found. The introduction of the second carbonyl group does not abolish, but slightly enhances, the shift to longer wavelengths caused by the first. This shift is reversed on dioxime formation, the spectrum however remaining of aetio type (dioximes of (III), (X) and (XI)). Two rhodofying groups on diagonally opposed pyrrole rings, e.g. rings I and III, however, enhance each other's rhodofying effect, and the oxorhodo-type spectrum results, as in (XXI) and (XXII). The former (XXI) was called by Fischer 'oxorhodoporphyrin'; its oxime has, as would be expected, not oxorhodo-type but rhodo-type spectrum. Unfortunately, this is the only example of this particular change on oxime formation in a compound of known structure.

A vinyl group diagonally opposed to a rhodofying group does not intensify the effect of the latter sufficiently to cause oxorhodo-type spectrum. Thus in (XX) the spectrum is rhodo type, not oxorhodo type, though band II is distinctly stronger than in the related ethyl compound (XIX).

These correlations are quite consistent among the simple porphyrins (i.e. excluding compounds containing the isocyclic ring which is characteristic of the chlorophyll derivatives). The oxime of chlorocruoroporphyrin (IX) was not studied by Stern, and according to non-quantitative published reports, had a rhodo-type spectrum, thus providing an apparent anomaly. It has, in fact, an aetio-type spectrum (Table 4).

Phaeoporphyrin a_5 (II) and phylloerythrin (IV), which contain isocyclic ring keto groups, require separate discussion. Their spectra are shown in Table 1. The cyclic, β-keto acid structure in phaeoporphyrin a_5 is very strongly rhodofying, the compound having a very marked oxorhodo-type spectrum. Phylloerythrin has lost the carboxyl group, and its spectrum is intermediate between rhodo type and oxorhodo type (band II ≏ band III); its ring keto group, while less strongly rhodofying than the carboxylated keto ring, exerts a much stronger effect in this direction than do keto groups in open chains attached to the pyrrole ring (cf. (XV), which has a true rhodo-type spectrum).

The oxime of phaeoporphyrin a_5 has a true rhodo-type spectrum, and that of phylloerythrin an aetio-rhodo type (band III ≏ IV).

It is thus apparent that the ring carboxyl group, which as written is not in conjugation with the nucleus, enhances the rhodofying effect of the ring keto group. Further, when the keto group is masked by oxime formation, the carboxyl group itself exerts a rhodofying effect. To explain this it is suggested that phaeoporphyrin a_5 and its oxime exist largely in the enol form.

Nothing is known about the equilibrium between these keto and enol forms, but the fact that on enolization the isocyclic ring becomes fully conjugated may favour the enol form.

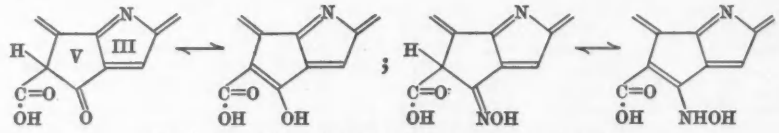


Fig. 2. Partial formulae of phaeoporphyrin a_5 and its oxime.

The fact that on enolization, (*a*) the carboxyl >C=O becomes conjugated with the nucleus, and (*b*) that ring V is brought into conjugation with the remainder of the porphyrin nucleus, may well explain the very strong oxorhodo-type spectrum of phaeoporphyrin a_5 and the rhodo-type spectrum of its oxime. Similarly, consideration of the additional resonance on enolization could explain not only the strong rhodo-type spectrum of phylloerythrin (band II ≏ III), but also the tendency towards rhodo type in its oxime (band III ≏ IV).

Possible structures for porphyrin a

As was found by Rawlinson & Hale (1949), porphyrin *a* has an oxorhodo-type spectrum, and its oxime is of rhodo type. From the knowledge of porphyrin compounds now available, this might be explained in three different ways:

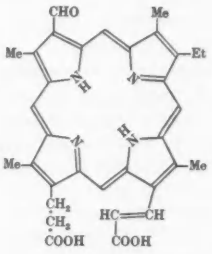
(1) The porphyrin has only one 'rhodofying' group. In this case the group should be a β-keto acid group in an isocyclic ring, as in phaeoporphyrin a_5 (II).

(2) There are two 'rhodofying' groups on opposite pyrroles, one of which should not be able to form an oxime (e.g. a carboxyl group as in (XXI) or an acrylic acid side chain as in (XVIII)).

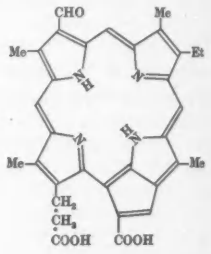
(3) There are two 'rhodofying' groups, one of which is a keto group in an isocyclic ring as in (1).

Such an assumption as (3) appears attractive, since compounds of this type are known in nature (bacteriochlorophyll), though in the tetrahydroporphin, not in the porphin series. Against assumption (1) is the fact that no compound of this type is known which has absorption bands as far shifted towards the red as has porphyrin *a*. Assumption (2) was supported not only on the ground of the similarity of the absorption spectra of porphyrin *a* and oxorhodoporphyrin (XXI), but also by the observation that the haemochromogen of oxorhodoporphyrin is so far the only haemochromogen of known structure which, like haemochromogen *a*, shows only a single absorption band. This single band is, moreover, in a position close to that of haemochromogen *a*. The iron complex of the 'formylrhodoporphyrin' (XXIII) might be expected to give a haemochromogen still more similar to that of haemin *a*.

However, evidence obtained by the London workers (cf. Rimington, Hale, Rawlinson, Lemberg & Falk, 1949) made such a hypothesis unlikely. They had found that oxime formation after reaction of porphyrin *a* with either diazoacetic ester or hydriodic acid caused the appearance of porphyrins of aetio type with shifts of band I of 17 and 19 mμ. respectively. A carboxylic acid group on a β-position on a pyrrole does not appear



XXIV



XXV

likely to be changed in these reactions, the evidence pointing rather to an ethylenic double bond. These reactions are also difficult to explain on the basis of assumption (3), since the keto group in the isocyclic ring does not react with hydriodic acid or diazoacetic ester. The only side chain with an ethylenic bond which is known to be rhodo-forming (as required by the rhodo-type spectrum of porphyrin *a* oxime) is the acrylic acid group, and the structure (XXIV), or possibly a similar compound containing a modified isocyclic nucleus (XXV), would be consistent with all evidence so far available. Other possibilities remain. The formula with one formyl group and one vinyl group in one ring and a vinyl group in the vicinal pyrrole ring, first suggested (without evidence) by Warburg (cf. Warburg, 1949) and independently by the London workers is also not excluded, since nothing is known about the spectroscopic type of porphyrins which contain vinyl and formyl on one ring. Although the vinyl group has usually no 'rhodofying' effect, it may possess it when on the same pyrrole ring as the aldehyde group in porphyrin *a*, or the aldoxime group in its oxime.

Finally, the behaviour of the haemochromogens to hydroxylamine and particularly to hydrazine (cf. Table 3) supplies reliable evidence that one of the 'rhodofying' groups is formyl and not an acetyl or an isocyclic ring keto group. The possibility that both a formyl and a keto group are present, on opposite pyrrole rings, is not excluded on the evidence from the haemochromogens alone, but such structures would not be consistent with the properties of porphyrin *a* and the products of its reactions with hydroxylamine, hydriodic acid and diazoacetic ester.

SUMMARY

1. The absorption spectra of haemin *a*, isolated from ox-heart muscle, and the related porphyrin *a* have been compared with those of a number of haemins and porphyrins of known structure.

2. The absorption spectra of the porphyrins of known structure and their oximes are completely in accordance with the correlations found by Stern and co-workers between structure and type of spectrum. Chlorocruoroporphyrin ('Spirographis' porphyrin) oxime, which, according to the literature, provided an exception, was shown in fact to be in accordance with these correlations. Though Negelein's 'cryptoporphyrin' from pigeon-breast muscle may well have been an artifact, we have not been able to confirm the particular evidence which led him to believe this.

3. The properties of the porphyrins and haemochromogens support previous evidence for the presence of an aldehyde (formyl) side chain in haemin *a*, and do not indicate the presence of a keto group in an isocyclic ring or in an open (e.g. acetyl) side chain.

4. Structures for porphyrin *a* which are consistent with all the evidence available, are those of 4-ethyl-2-formyl-1:3:5:8-tetramethylporphin-6-acrylic acid-7-propionic acid, or the same compound where the γ-methene-bridge carbon and the α-carbon of the acrylic acid are joined with loss of two H atoms to form a five-membered ring. More direct evidence upon the structure of the natural compound is, however, required.

One of us (J.E.F.) was the holder at the Institute of Medical Research, Royal North Shore Hospital of Sydney, of a Fellowship from the National Health and Medical Research Council of Australia during part of this work, and later of a Nuffield Fellowship at the University of London. We thank Prof. C. Rimington, Department of Chemical Pathology, University College Hospital Medical School, London, for his kindness in allowing one of us (J.E.F.) to complete in his Department some work begun in Sydney. We also thank Prof. W. A. Rawlinson for a sample of porphyrin *a*, Dr J. H. Hale for a sample of chlorocruorohaemin and Prof. S. Goldschmidt for samples of monoacetyldeuteroporphyrin and of phaeoporphyrin a_5 dimethyl ester from the collection of the late Prof. H. Fischer. The Beckman spectrophotometer was purchased out of a grant to Prof. Rimington from the Central Research Funds Committee of the University of London.

REFERENCES

Eigenberger, E. (1931). *J. prakt. Chem.* **130**, 75.
Fischer, H. & Beer, L. (1936). *Hoppe-Seyl. Z.* **244**, 31.
Fischer, H. & Deilmann, K. (1944). *Hoppe-Seyl. Z.* **280**, 186.
Fischer, H. & Krauss, G. (1936). *Liebigs Ann.* **521**, 270.
Fischer, H. & Mittermair, J. (1941). *Liebigs Ann.* **548**, 147.
Fischer, H. & Seemann, C. v. (1936). *Hoppe-Seyl. Z.* **242**, 133.
Fischer, H. & Wecker, G. (1942). *Hoppe-Seyl. Z.* **272**, 1.
Fischer, H. & Zeile, K. (1929). *Liebigs Ann.* **468**, 99.
Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
Lemberg, R., Falk, J. E., Rimington, C., Hale, J. H. & Rawlinson, W. A. (1949). 1*st Int. Congr. Biochem.* Abstr. p. 351.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*, p. 360. New York: Interscience.
Negelein, E. (1932*a*). *Biochem. Z.* **248**, 243.
Negelein, E. (1932*b*). *Biochem. Z.* **250**, 577.
Negelein, E. (1933). *Biochem. Z.* **266**, 412.
Rawlinson, W. A. & Hale, J. H. (1949). *Biochem. J.* **45**, 247.
Rimington, C., Hale, J. H., Rawlinson, W. A., Lemberg, R. & Falk, J. E. (1949). 1*st Int. Congr. Biochem.* Abstr. pp. 351, 378, 379.
Roche, J. & Bénévent, M. T. (1936). *Bull. Soc. Chim. biol., Paris*, **18**, 1650.
Schumm, O. (1928). *Hoppe-Seyl. Z.* **178**, 11.
Stern, A. & Molvig, H. (1936). *Z. phys. Chem.* A, **177**, 365.
Stern, A. & Wenderlein, H. (1934). *Z. phys. Chem.* A, **170**, 345.
Stern, A. & Wenderlein, H. (1935*a*). *Z. phys. Chem.* A, **174**, 81.
Stern, A. & Wenderlein, H. (1935*b*). *Z. phys. Chem.* A, **174**, 321.
Stern, A. & Wenderlein, H. (1935*c*). *Z. phys. Chem.* A, **175**, 405.
Stern, A. & Wenderlein, H. (1936). *Z. phys. Chem.* A, **176**, 81.
Warburg, O. (1949). *Heavy Metal Prosthetic Groups and Enzyme Action*. Oxford: Clarendon Press.
Warburg, O. & Negelein, E. (1932). *Biochem. Z.* **244**, 239.
Warburg, O., Negelein, E. & Haas, E. (1930). *Biochem. Z.* **227**, 171.

An Improved Procedure for the Determination of Carbon in Organic Compounds in Aqueous Solution

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The rapid semi-micro determination of carbon by wet combustion of organic compounds (Van Slyke & Folch, 1940) has been subjected to various modifications, principally in the apparatus used (McCready & Hassid, 1942; Hockenhull, 1950).

The apparatus is shown in Fig. 1, and is essentially that described by Hockenhull (1946; and personal communication), using the spiral bubbler modified from that of Pearce & Robertson (1936).

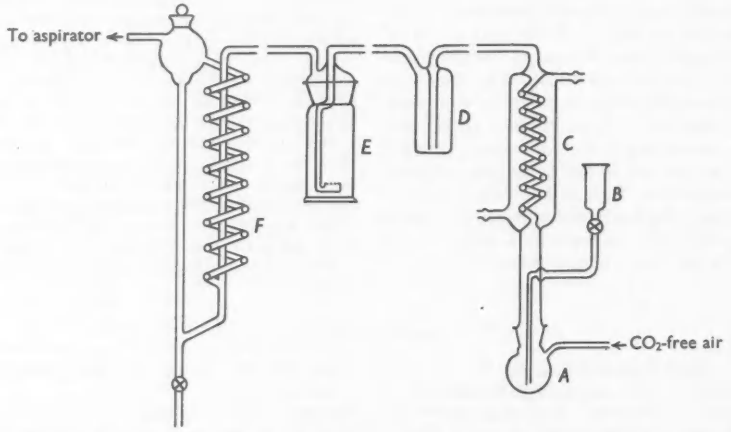

Fig. 1. Apparatus for the determination of carbon in compounds in aqueous solution. Scale = $\frac{1}{3}$. Constructed in thick-wall Pyrex glass. The ground joint connecting the combustion flask (*A*) to the rest of the apparatus is size B19. (*A*) is the combustion flask, (*B*) is the dropping funnel, (*C*) is a water condenser. Bubbler (*D*) contains pure H_2SO_4. Bubbler (*E*) contains water acidified with H_2SO_4. The spiral bubbler (*F*) contains barium hydroxide. The sections of the apparatus are joined together by rubber tubing.

The application of this procedure to aqueous samples presents a difficulty, since the combustion is then incomplete (Van Slyke & Folch, 1940). Friedeman & Kendal (1929) had previously overcome this difficulty by working on a macro scale and using large volumes of concentrated sulphuric acid to absorb the excess water. In this way the carbon of ethanol could be directly estimated. The need for a semi-micro method for the rapid analysis of total carbon in fermentation liquids prompted the following modification of the method of Hockenhull (1950).

Water is removed from the reaction mixture by excess phosphorus pentoxide in the combustion flask before combustion.

METHOD

Reagents. Oxidizing mixture (Van Slyke & Folch, 1940); P_2O_5; KIO_3 (A.R.); 0·1 N-HCl; 0·05 N-$Ba(OH)_2$; phenolphthalein (1% w/v in ethanol); *n*-butanol (redistilled).

Apparatus and procedure. In the combustion flask (*A*) is placed P_2O_5 (1·5 g./ml. of liquid in the sample) and 200 mg. of KIO_3. The joints of *A* and *B* are moistened with syrupy orthophosphoric acid and the flask is fitted to the apparatus. The oxidizing mixture (5 ml.) is then added and CO_2-free air is aspirated for 5 min. The tip of the dropping funnel (*B*) must be below the surface of the oxidizing mixture at this stage, otherwise when the sample is added loss will occur due to volatilization of labile constituents. After 5 min. the air flow is stopped and 40–50 ml. of 0·05 N-$Ba(OH)_2$ (exactly measured), together with 2–3 ml. of *n*-butanol and 0·5 ml. of

Table 1. *Carbon content of various substances determined in aqueous solution*

(Glucose was British Drug Houses Analar dextrose. *iso*Butyric acid was supplied by Mr K. el Shazly; uric acid was a specimen from Kahlbaum. The purity of the ethanol (Distillers Company Ltd.) was estimated by dichromate oxidation.)

Substance	Vol. of solution (ml.)	Wt. of substance taken (mg.)	Carbon content				P_2O_5 (g.)
			Calculated (mg.)	Found (mg.)	Loss or gain (mg.)	Recovery (%)	
Glucose	1	14·99	5·99	5·94	−0·05	99·4	1·5
	1	14·99	5·99	6·07	+0·08	100·5	
	1	14·99	5·99	6·13	+0·14	101·2	
	4	14·99	5·99	5·94	−0·05	99·4	5–6
	4	14·99	5·99	5·87	−0·12	98·6	
	4	14·99	5·99	6·07	+0·08	100·5	
*iso*Butyric acid	1	8·626	4·70	4·95	+0·25	105·0	1·5
	1	8·626	4·70	5·01	+0·31	106·1	
	1	8·684	4·73	4·68	−0·05	98·9	
	1	8·684	4·73	4·81	+0·07	100·5	
	1	8·684	4·73	4·62	−0·11	98·3	
Ethanol	1	9·145	4·77	4·62	−0·15	96·8	1·5
	1	9·145	4·77	4·55	−0·22	95·4	
	1	9·145	4·77	4·62	−0·15	96·8	
	4	9·145	4·77	4·75	−0·02	99·5	5–6
	4	9·145	4·77	4·58	−0·09	98·1	
Uric acid	2	11·98	4·26	4·42	+0·16	103·7	3–4
	2	11·98	4·26	4·35	−0·09	102·1	

phenolphthalein solution, are added to the spiral bubbler. The *n*-butanol prevents foaming, assists aggregation of particles of $BaCO_3$ and further forms a protective surface layer during the final titration. The aqueous sample (containing about 4 mg. C) is then carefully added and washed in with 2 ml. of oxidizing mixture. CO_2-free air is again aspirated through the apparatus while the contents of the flask (*A*) are heated until refluxing freely. After 15 min. the heating is stopped and the contents of the spiral bubbler are run into a 250 ml. flask. A three-way stopcock on the aspirator bottle allows the apparatus to be open to the air during this operation. The spiral is rinsed out by filling with CO_2-free water and again running out; the total liquid is then titrated with 0·1 N-HCl. A blank is run under similar conditions. 1 ml. 0·1 N-HCl ≡ 0·60 mg. C.

RESULTS

The results of the analysis of four compounds are shown in Table 1. For samples containing about 5 mg. carbon the recoveries range from 95 to 106 %; the procedure is, therefore, sufficiently accurate for many biological purposes, for example, the determination of carbon in fermentation liquids. In this latter respect the high recoveries obtained with ethanol are of particular interest.

SUMMARY

1. A method is described for the rapid determination of carbon in aqueous solutions of organic compounds.

2. For samples containing about 5 mg. carbon, the method is accurate within ± 5 %.

I wish to thank the Faculty of Technology, The University, Manchester, for a gift of the apparatus, Mr K. Bartholomew, glass-blower, for constructing it, and Mr K. el Shazly, Rowett Research Institute, for providing a highly purified sample of *iso*butyric acid.

REFERENCES

Friedeman, T. E. & Kendall, A. I. (1929). *J. biol. Chem.* **82**, 45.
Hockenhull, D. J. D. (1946). *Biochem. J.* **40**, 337.
Hockenhull, D. J. D. (1950). *Biochem. J.* **46**, 605.
McCready, R. M. & Hassid, W. Z. (1942). *Industr. Engng Chem.* (Anal. ed.), **14**, 525.
Pearce, P. H. & Robertson, E. C. (1936). *Chem. & Ind.* **55**, 543.
Van Slyke, D. D. & Folch, J. J. (1940). *J. biol. Chem.* **136**, 509.

The Oxidation in Liver of L-Tyrosine to Acetoacetate through *p*-Hydroxyphenylpyruvate and Homogentisic Acid

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The picture of L-tyrosine metabolism obtained from isotope experiments and other studies on intact animals is still incomplete. Either *p*-hydroxyphenylpyruvate or 2:5-dihydroxyphenylalanine may be the first intermediate, since either could form the second postulated intermediate, 2:5-dihydroxyphenylpyruvate (Neubauer, 1909), and both are converted to homogentisic acid by alcaptonurics (Neuberger, Rimington & Wilson, 1947). *p*-Hydroxyphenylpyruvate is also known to be formed from tyrosine in scorbutic guinea pigs and premature infants (Sealock & Silberstein, 1940; Levine, Marples & Gordon, 1941) and was excreted by Medes's case of tyrosinosis (Medes, 1932). However, the evidence for the assumption that normal tyrosine metabolism goes through these homogentisic acid precursors and homogentisic acid itself is only indirect. Normal liver can oxidize homogentisic acid to acetoacetate (Edson, 1935*b*; Zorn, 1940; Ravdin & Crandall, 1950). Still more significant is the isotopic labelling of acetoacetate formed from labelled phenylalanine by liver slices (Schepartz & Gurin, 1949; Weinhouse & Millington, 1948), which shows that a molecular rearrangement of the side chain must have occurred. This rearrangement is similar to that which must be postulated for the intermediate formation of homogentisic acid from tyrosine (Meyer, 1901; Friedman, 1908).

Investigations of the oxidation of L-tyrosine in liver homogenates have not resolved any of the uncertainties about the reaction. There is agreement that the oxidation of L-tyrosine by liver homogenates uses four atoms of oxygen and produces one molecule each of carbon dioxide and acetoacetate but no ammonia (Bernheim & Bernheim, 1934; Felix, Zorn & Dirr-Kaltenbach, 1937; Zorn, 1940; Felix & Zorn, 1941; Sealock & Goodland, 1949). In liver slices fumaric acid (or malic acid) and acetoacetate are formed from L-tyrosine (Lerner, 1949). Felix & Zorn (1941) also found alanine to be a product of the reaction, and they thought that this arose from a splitting-off of the tyrosine side chain. Isotope experiments have failed to confirm this origin of the alanine (Lerner, 1949). The most thorough investigations of the intermediate steps (Felix *et al.* 1937; Zorn, 1940) have apparently excluded both *p*-hydroxyphenylpyruvate and homogentisic acid as intermediates. Their investigations suggest a totally different pathway of L-tyrosine oxidation in liver than that deduced from intact animal experiments.

A more active L-tyrosine oxidation system than any hitherto used, freed from other reactions occurring in crude liver homogenates, was needed to investigate these discrepancies. Concentrated suspensions of liver homogenates have always been used to obtain the highest tyrosine oxidation activity (Bernheim & Bernheim, 1934; Sealock & Goodland, 1949), presumably to avoid dilution of unidentified dialysable components of the system. Using such suspensions, Rienits (1950) and Painter & Zilva (1950) have shown that addition of ascorbic acid gave a small but statistically significant increased oxidation of L-tyrosine in liver preparations from both scorbutic and normal guinea pigs. The recent identification of α-ketoglutarate and ascorbic acid as the major co-factors for L-tyrosine oxidation in liver homogenates (LeMay-Knox & Knox, 1951) has permitted a higher order of activity to be obtained. These additions to the reaction have also made possible some purification of the system and almost complete elimination of secondary reactions. With such preparations the pathway of tyrosine oxidation in liver has been re-investigated.

METHODS

Enzyme preparation. Rat livers were promptly chilled after removal from the animal and homogenized in a Waring blender with 3 parts of ice-cold 0·9% KCl, following the procedure used previously (Knox & Mehler, 1950). The soluble enzyme system was obtained free of mitochondrial elements by centrifuging at 6000 *g* for 20 min. and was then dialysed against tap water overnight. The preparations retained their activity for several days at 2°.

Enzyme reactions and assays. The oxidations were carried out in manometer cups or open flasks shaken in a water bath at 37° with air as the gas phase. The reaction mixture contained 1 ml. of the enzyme preparation and 1 ml. of 0·2M-phosphate, pH 7·5, in a total volume of 3 ml. When tyrosine and *p*-hydroxyphenylpyruvate were substrates, ascorbic acid was added, usually 0·1 ml. of a freshly made up and neutralized 0·5% solution. L-Tyrosine (1·5 mg./ml.) was dissolved in the buffer with heating, and the reaction started by tipping in from a side arm 0·3 ml. of 0·1M-α-ketoglutarate. The transaminase reaction was carried out in a mixture similar to that used for tyrosine oxidation, but without

ascorbic acid and under an atmosphere of N_2. The *p*-hydroxyphenylpyruvate formation was followed in the Beckman spectrophotometer at 1 min. intervals by the increase in light absorption at 320 mμ., or it was determined chemically after 20 min. incubation.

Analytical methods

The disappearance of *p*-hydroxyphenyl compounds was determined in 5% (w/v) metaphosphoric acid filtrates of the initial and final reaction mixtures by the Millon reaction, following the conditions of Medes (1932). The final colour, produced in the same degree by identical molar concentrations of tyrosine and *p*-hydroxyphenylpyruvate and not by the 2:5-dihydroxyphenyl compounds (Neuberger, 1947), was measured in the spectrophotometer at 480 mμ. *p*-Hydroxyphenylpyruvate was determined chemically in 5% (w/v) trichloroacetic acid filtrates by the Briggs reaction (Neuberger, 1947). In the spectrophotometric assay at 320 mμ. in 1 cm. cells, a change in optical density of 0·810 corresponds to 1·0 μmol./ml. of *p*-hydroxyphenylpyruvate.

Acetoacetic acid was determined manometrically with aniline citrate (Edson, 1935 *a*). L-Glutamic acid was determined by the specific decarboxylase method of Gale (1945), using suspensions of *Clostridium welchii* provided by Miss van Haltern.

The 2:4-dinitrophenylhydrazone of *p*-hydroxyphenylpyruvate was isolated from a trichloroacetic acid filtrate of a large-scale anaerobic reaction mixture by the procedure of Stumpf & Green (1944). The hydrazone, after chromatography in ethyl acetate on an alumina column (Datta, Harris & Rees, 1950) to separate the slower-moving hydrazone of unreacted α-ketoglutarate, was recrystallized from ethyl acetate and light petroleum.

The enzyme system oxidizing homogentisic acid was inactivated by adding 0·5M-NaH_2PO_4 containing 1% (v/v) acetic acid to pH 5, and after 15 min. at room temperature the mixture was neutralized to pH 7·2. For the isolation of homogentisic acid 50 ml. of a solution of this treated enzyme were incubated with tyrosine, α-ketoglutarate and ascorbic acid. The reaction was followed in a pilot run, and when this indicated completion of the reaction the mixture was deproteinized with metaphosphoric acid. Continuous extraction of the filtrate with peroxide-free ether, concentration and extraction back into water, taking advantage of the favourable partition of homogentisic acid (Neuberger, 1947), was repeated twice for purification. The solution obtained was chromatographed (descending solvent) using both *n*-butanol-water with a few drops of formic acid in the cabinet to prevent tailing, and the solvent C80 of Smith (1950) which consists of 80% saturated $(NH_4)_2SO_4$ containing 2% (v/v) *iso*propanol. The spots were located after 10 hr. runs by spraying with ammoniacal $AgNO_3$ (Bray, Thorpe & White, 1950). In *n*-butanol-water the isolated product and an authentic sample of homogentisic acid had the same R_F value of 0·5–0·6, very close to that of gentisic acid, but easily separated from *p*-hydroxyphenylpyruvic acid (R_F 0·1) and 2:5-dihydroxyphenylpyruvic acid (two spots, R_F 0·2 and 0·8). In solvent C80 the product and homogentisic acid had an R_F of 0·7 while gentisic acid was well separated (R_F 0·3) and the two keto acids less so (R_F values 0·60–0·65). The gentisic acid, but not the homogentisic acid spot, fluoresced in ultraviolet light.

Substrates. The compounds *p*-hydroxyphenylpyruvate, L-2:5-dihydroxyphenylalanine, the lactone of 2:5-dihydroxyphenylpyruvic acid, and homogentisic acid, without which this work could not have been done, were very kindly provided by Dr A. Neuberger. The lactone of 2:5-dihydroxyphenylpyruvic acid was hydrolysed to the free acid by the procedure of Neubauer & Flatow (1907), and neutral solutions of this acid were made up under N_2 immediately before use. Despite the lability of these compounds to oxidation, controls revealed no spontaneous oxidation nor was there darkening of the enzyme reaction mixtures containing them in the experiments at pH's of 7·5 or lower.

Sodium gentisate was obtained from Dr Severinghaus of Hoffman-LaRoche, Inc. Calcium pyridoxal-phosphate was a gift from Dr W. Umbreit of Merck and Co. D-*iso*Ascorbic acid was obtained from Dr F. Bergel of Roche Products Ltd.

RESULTS

The oxidation of L-tyrosine in the presence of α-ketoglutarate and ascorbic acid

The oxygen uptake and L-tyrosine disappearance catalysed by rat-liver homogenates was increased approximately tenfold by additions of α-ketoglutarate and ascorbic acid (LeMay-Knox & Knox, 1951). The full activity of the homogenates was also obtained in the dialysed soluble fraction of liver used in the present studies when it was supplemented with both α-ketoglutarate and ascorbic acid. Little or no activity was found unless both compounds were added together (Figs. 1 and 2). Addition of these compounds also increased tyrosine oxidation in similar liver fractions from guinea pigs and rabbits.

The complete system will take up approximately four atoms of oxygen and form one molecule of acetoacetate for each molecule of L-tyrosine oxidized (Table 1). The use of four atoms of oxygen instead of the theoretical value of five (to form acetoacetate and fumarate), and the requirement for α-ketoglutarate, suggest that a transaminase reaction replaces the oxidative deamination. This was confirmed by the formation of L-glutamate during the reaction (Table 1).

Table 1. *Acetoacetate and glutamate formation during L-tyrosine oxidation by the rat-liver enzyme*

(1 ml. enzyme solution; 1 ml. 0·2M-phosphate, pH 7·5; 0·5 mg. ascorbic acid; 30 μmol. α-ketoglutarate; approx. 1·5 mg. L-tyrosine. Incubation: 1 hr. at 38°.)

L-Tyrosine disappearing (μmol.)	Oxygen consumed (μmol.)	Acetoacetate formed (μmol.)	L-Glutamate formed (μmol.)
8·4	17·1	8·4	—
10·1	—	10·3	—
—	13·0	6·2	—
7·1	—	7·0	6·3
6·6	—	5·5	6·5

—, not determined.

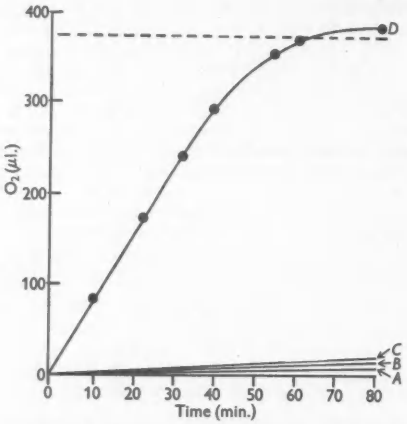

Fig. 1. Effect of addition of α-ketoglutarate on the oxidation of L-tyrosine. All vessels contained 1 ml. of enzyme solution, 1 ml. 0·2 M-phosphate, pH 7·5, and 0·5 mg. ascorbic acid, in a total volume of 3·0 ml. *A*, without additions; *B*, +8·4 μmol. of L-tyrosine; *C*, +30 μmol. α-ketoglutarate; *D*, + both L-tyrosine and α-ketoglutarate. – – – theoretical O_2 uptake of 4 atoms/mol. of tyrosine.

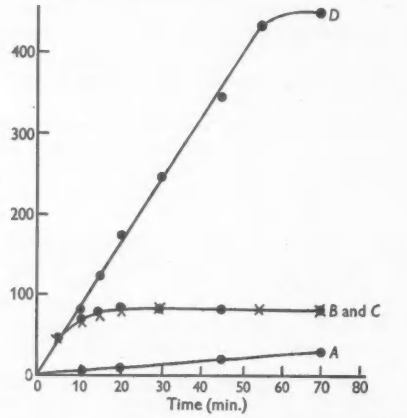

Fig. 2. Effect of additions of ascorbic acid and of glutathione on the oxidation of L-tyrosine. All vessels contained enzyme solution and phosphate as in Fig. 1, and 30 μmol. of α-ketoglutarate. *A*, +0·1 μmol. of ascorbic acid; *B* (●), +10 μmol. of L-tyrosine; *C* (×), +L-tyrosine and 1 mg. of glutathione; *D*, +L-tyrosine and ascorbic acid. The O_2 uptakes of enzyme alone and enzyme + glutathione (not shown) and of enzyme + ascorbic acid (*A*) have been subtracted respectively from curves *B*, *C* and *D*.

Pyruvate could replace α-ketoglutarate in a crude homogenate (LeMay-Knox & Knox, 1951), but not in the dialysed soluble enzyme unless a small amount of α-ketoglutarate was also added. When tyrosine was oxidized in this way with pyruvate present, the accumulation of alanine instead of glutamate could be demonstrated chromatographically.

Transamination reaction

The absence of oxygen uptake or any diminution in the Millon reaction with the enzyme plus tyrosine, unless α-ketoglutarate is present, suggests that the transamination of tyrosine is an obligatory initial reaction. An initial oxidation of tyrosine to 2:5-dihydroxyphenylalanine has been directly excluded in the dialysed soluble system, since L-2:5-dihydroxyphenylalanine in contrast to *p*-hydroxyphenylpyruvate was not oxidized in this system.

A tyrosine-glutamic transaminase similar to that of Hird & Rowsell (1950) and Cammarata & Cohen (1950) was detected in liver. The activity was found to be distributed nearly equally between the insoluble and soluble fractions of liver obtained by high-speed centrifugation. The reaction in the soluble fraction could be separated from the subsequent oxidation steps of tyrosine under anaerobic conditions. The transamination of L-tyrosine proceeded at a linear rate proportional to the concentration of the enzyme until over half of the added α-ketoglutarate was converted to glutamate. *p*-Hydroxyphenylpyruvate, also formed by the reaction, was identified by isolation as the 2:4-dinitrophenylhydrazone.

Dialysed liver supernatant (15 ml.) was incubated for 2 hr. under N_2 with 29 ml. of 0·1 M-phosphate, pH 7·2, containing 37 mg. of L-tyrosine and 29 mg. of α-ketoglutarate. The *p*-hydroxyphenylpyruvate formed (27 mg.) as determined colorimetrically represented a 75% conversion of the α-ketoglutarate to glutamate. The recrystallized 2:4-dinitrophenylhydrazone obtained had a m.p. 170–171°. The mixed m.p. with an authentic sample (m.p. 171°), prepared in the same way, was 170–171° (all m.p.'s uncorr.).

If transamination is the obligatory initial reaction of L-tyrosine oxidation, the rate of transamination, if sufficiently slow, should determine the rate of the overall oxidation of tyrosine. The rate of transamination, generally faster than the overall tyrosine oxidation in fresh enzymes, could be decreased by certain treatments. Dialysis, or better, ammonium sulphate precipitation followed by dialysis, partially removed the coenzyme of the transaminase. After such treatments the slower rate of the transaminase reaction did limit the rate of the aerobic tyrosine oxidation reaction (Fig. 3). The transaminase activity lost by such treatments could be largely restored by addition of boiled fresh enzyme or by addition of pyridoxal phosphate. The addition of pyridoxal phosphate to an enzyme in which the transaminase reaction was the limiting step of tyrosine oxidation increased the rate of the transamination reaction, and by the same effect, increased the rate of the tyrosine oxidation (Fig. 4).

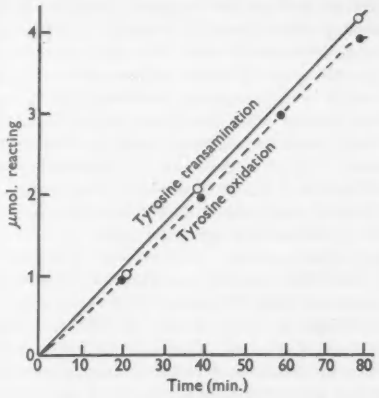

Fig. 3. Limitation of the rate of L-tyrosine oxidation by the rate of tyrosine transamination. All vessels contained 1 ml. of enzyme solution which had been precipitated between 30 and 50% saturation with $(NH_4)_2SO_4$ and dialysed, 1 ml. 0·2 M-phosphate, pH 7·5, 20 μmol. α-ketoglutarate and 8·5 μmol. L-tyrosine, in 3·2 ml. total volume. Anaerobic formation of *p*-hydroxyphenylpyruvate (○), determined by the Briggs reaction, +0·5 mg. of ascorbic acid, and the aerobic disappearance of tyrosine (and *p*-hydroxyphenylpyruvate) measured by the Millon reaction (●).

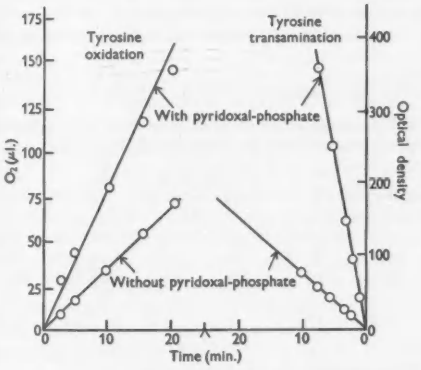

Fig. 4. Effect of pyridoxal phosphate on the rates of tyrosine transamination and oxidation. Tyrosine oxidation (left, ○) was determined manometrically with an enzyme solution, and additions the same as in Fig. 3. Tyrosine transamination (right, ○) was determined in a similar system, but with one-half the enzyme concentration (0·5 ml. in 3·2 ml. total vol.), by the anaerobic formation of *p*-hydroxyphenylpyruvate measured at 320 mμ. The optical density has been drawn to twice the scale of the O_2 uptake. Pyridoxal phosphate (50 μg.) added as indicated.

Ascorbic acid requirement for p-hydroxyphenylpyruvate oxidation

Ascorbic acid has no effect on the initial deamination of tyrosine to *p*-hydroxyphenylpyruvate and is required only for the next step, the oxidation of *p*-hydroxyphenylpyruvate (Fig. 2). In some preparations, like that used in Fig. 2, as little as 0·1 μmol. of ascorbic acid permitted the oxidation of an additional 5 μmol. or more of tyrosine, i.e. an amount 50 times the equivalent of that of the added ascorbic acid. The amount of ascorbic acid required for full activity of the system varied somewhat in different preparations. Generally, more ascorbic acid was required to obtain the same activity before dialysis than after dialysis (see Table 2). This catalytic effect of ascorbic acid on the oxidation of tyrosine is specific in so far as it was not shown by other reducing agents such as glutathione, cysteine, or dihydroxymaleic acid. Ascorbic acid can also form peroxide, but peroxide generated during the reaction by notatin and glucose will not replace the ascorbic acid requirement. Folic acid, which is reported to restore tyrosine metabolism to normal in scorbutic guinea pigs (Woodruff & Darby, 1948), is also inactive. *iso*Ascorbic acid had the same activity as ascorbic acid (Table 2).

Table 2. *Comparison of the effects of* D-*ascorbic and* D-iso*ascorbic acids on* L-*tyrosine oxidation*

(Experimental details as in Table 1 except for ascorbic acid additions. Tyrosine disappearance was measured after 1 hr. although the reactions with suboptimal amounts of ascorbic or *iso*ascorbic acid did not necessarily continue for this length of time (cf. Fig. 2).)

Substance and amount (mg./3 ml.)	L-Tyrosine oxidized (μmol. in 1 hr.)	
	Undialysed enzyme	Dialysed enzyme
— —	2·95	2·30
Ascorbic acid 0·02	—	2·29
*iso*Ascorbic acid 0·02	—	2·35
Ascorbic acid 0·05	3·96	—
*iso*Ascorbic acid 0·05	4·00	—
Ascorbic acid 0·10	4·97	9·46
*iso*Ascorbic acid 0·10	5·13	9·67
Ascorbic acid 0·30	8·97	—
*iso*Ascorbic acid 0·30	8·49	—
Ascorbic acid 0·50	9·34	9·66
*iso*Ascorbic acid 0·50	9·58	9·65

—, not determined.

In the absence of ascorbic acid an initial oxidation of tyrosine to acetoacetate nearly always occurred, and at a rate equal to that with ascorbic acid. However, the reaction without ascorbic acid soon stopped abruptly. With suboptimal amounts of ascorbic acid longer periods of reaction at the initial

rate were obtained, and with optimal amounts of ascorbic acid the reaction continued at the same rate for several hours. Ascorbic acid and *iso*ascorbic acid appeared to prolong tyrosine oxidation rather than to increase the rate of the reaction.

Pathway of p-*hydroxyphenylpyruvate oxidation to acetoacetate*

The system oxidizing L-tyrosine will not oxidize tyramine, L-2:5-dihydroxyphenylalanine, the lactone of 2:5-dihydroxyphenylpyruvic acid or gentisic acid. Several possible reaction pathways are therefore eliminated, and a degree of specificity can be attributed to the system. This specificity increases the probability that the compounds which can be oxidized by the system, *p*-hydroxyphenylpyruvic acid, 2:5-dihydroxyphenylpyruvic acid and homogentisic acid, are indeed successive intermediates in L-tyrosine oxidation. These compounds are all oxidized to acetoacetate with the theoretical oxygen uptakes and at rates comparable with that of the overall oxidation of L-tyrosine (Table 3).

The identification of *p*-hydroxyphenylpyruvate formed from L-tyrosine by the transaminase in this enzyme preparation has already been described.

The system in the enzyme preparation which oxidizes homogentisic acid can be preferentially inactivated while the initial tyrosine oxidation reactions are preserved. Losses of the homogentisic acid oxidizing activity noted during fractionation procedures could be attributed to the low pH produced in ammonium sulphate solutions during dialyses (Gilbert & Swallow, 1950). It was more convenient to expose the enzyme to pH 5 at room temperature for 15 min. This inactivated the system oxidizing homogentisate to acetoacetate and, though the initial tyrosine oxidation reactions were reasonably well preserved, the formation of acetoacetate from tyrosine was also stopped (Table 4). In this way, preparations were readily obtained that catalysed only two steps of the oxidation of tyrosine, the further oxidation to acetoacetate being eliminated simultaneously with the suppression of the system oxidizing homogentisic acid.

The partial oxidation of tyrosine by an enzyme treated at pH 5 yielded a product which resembled homogentisic acid. It had an ultraviolet absorption curve similar to that of the 2:5-dihydroxyphenyl compounds, darkened when shaken with air in the presence of alkali, and reduced ammoniacal silver nitrate in the cold. This product was isolated from two large-scale runs by continuous ether extraction and partition between water and ether, and then chromatographed in two different solvents. The product moved in a single spot identically with authentic homogentisic acid in both solvents, and could be separated from gentisic acid, *p*-hydroxyphenylpyruvic acid and 2:5-dihydroxyphenylpyruvic acid.

DISCUSSION

It is possibly fortuitous that all of the enzymes in liver catalysing the successive reactions of L-tyrosine to acetoacetate occur in the soluble fraction. The same overall reaction occurring in the homogenate was obtained intact with this fraction alone,

Table 3. *Oxidation of proposed intermediates by the* L-*tyrosine oxidizing system*

(Experimental details generally similar to those described in Table 1. In experiments with *p*-hydroxyphenylpyruvate no α-ketoglutarate, and in experiments with 2:5-dihydroxyphenylpyruvate and homogentisate no α-ketoglutarate and no D-ascorbic acid was added.)

	L-Tyrosine		*p*-Hydroxyphenylpyruvic acid			2:5-Dihydroxyphenylpyruvic acid		Homogentisic acid	
Exp. no.	1	2	1	2	3	1	2	1	2
Rates of oxidation (μl. O_2/ml. enzyme/first 10 min.)	55	49	53	51	70	32	37	50	80
Atoms O_2/mol. acetoacetate formed	4·3	4·2	3·8	4·2	4·0	3·1	3·2	2·1	2·1

Table 4. *Elimination of the formation of acetoacetate from* L-*tyrosine by destroying the system oxidizing homogentisic acid*

	Substrate			
	L-Tyrosine		Homogentisic acid	
Treatment of enzyme	Oxygen uptake (μmol./30 min./ml.)	Acetoacetate formed (μmol./30 min./ml.)	Oxygen uptake (μmol./30 min./ml.)	Acetoacetate formed (μmol./30 min./ml.)
Dialysed	21·6	5·2	13·4	6·4
Dialysed and exposed to pH 5 for 15 min.	5·9	<0·1	0·3	<0·1

and its analysis was greatly simplified by even this degree of purification. The separation of the mitochondria avoided the further metabolism of the products, acetoacetate and fumarate (see below), and of α-ketoglutarate and glutamic acid. With moderate dialysis of the supernatant it was possible to reduce the blank reactions and to demonstrate clearly the requirements for α-ketoglutarate and ascorbic acid. It will be desirable, now that the overall reaction has been clarified, to have more detailed analyses of the individual steps than was possible in the preparations used here.

The first reaction, the deamination of L-tyrosine to *p*-hydroxyphenylpyruvate with its transaminase, is the most clearly characterized. It is an obligatory initial reaction in the L-tyrosine oxidation as determined by the absence of any reaction without α-ketoglutarate, and by the failure of the possible alternative intermediate, 2:5-dihydroxyphenylalanine, to be metabolized in the system. The formation of *p*-hydroxyphenylpyruvate from L-tyrosine by the transaminase in the system has been demonstrated, as has the further oxidation of this keto acid. The further reaction of *p*-hydroxyphenylpyruvate is the same as that of tyrosine, that is, both form the same product, acetoacetate, and at comparable rates. The ultraviolet absorption of this aromatic keto acid provides a convenient measure of the transamination, a method which may be of more general application. Additional treatments of the enzyme preparation, such as ammonium sulphate precipitation and/or dialysis, partially remove the coenzyme of this tyrosine-glutamic acid transaminase. The activity can then be restored by addition of pyridoxal phosphate, in agreement with the results of Cammarata & Cohen (1950), and of Feldman & Gunsalus (1950) for the bacterial enzyme. Liver preparations, partially deficient in pyridoxal phosphate, were used by adding or withholding the coenzyme to demonstrate that the rate of the transaminase reaction does, in fact, control the rate of the overall oxidation of tyrosine. Only the transaminase reaction occurs in the system anaerobically and substantially only this reaction in the absence of ascorbic acid aerobically. *p*-Hydroxyphenylpyruvate then accumulates, as it does in scorbutic guinea pigs fed tyrosine.

The oxidation of *p*-hydroxyphenylpyruvate does not take place in the side chain, since the product, *p*-hydroxyphenylacetate, is not a precursor of homogentisic acid (Neubauer, 1904). The oxidation, in the ring, must form 2:5-dihydroxyphenylpyruvate. This unusual reaction involves a migration of the pyruvyl side chain, in addition to the oxidation of the ring, and is of interest apart from the catalytic action of ascorbic acid in this step. The oxidation of *p*-hydroxyphenylpyruvate with an oxygen uptake of three atoms per mol. instead of four, and the formation of 0·67 mol. of acetoacetate instead of one (Felix *et al.* 1937), was apparently due to incomplete oxidation.

2:5-Dihydroxyphenylpyruvate is oxidized by the system to the same product and at a similar rate to L-tyrosine. However, no attempt was made to accumulate this compound from the reaction of L-tyrosine or *p*-hydroxyphenylpyruvate, a necessary step before it is definitely identified as an intermediate. Oxidative decarboxylation of this probable intermediate would give rise to the carbon dioxide formed during tyrosine oxidation and to homogentisic acid.

Homogentisic acid was identified with reasonable certainty as the product formed by the oxidation of L-tyrosine with two atoms of oxygen and accumulated when the enzyme oxidizing homogentisic acid was destroyed. This compound, previously known to be formed only in alcaptonurics and in certain abnormal states, can now be accepted as a normal intermediate in tyrosine metabolism. It is oxidized to acetoacetate by liver at a more rapid rate than is tyrosine, and ascorbic acid is not required. Two enzymes responsible for the disappearance of homogentisic acid in liver have been identified (Ravdin & Crandall, 1950). One oxidizes the homogentisic acid to fumaryl-acetoacetate, and the second hydrolyses this new intermediate to fumaric acid and acetoacetate. It has also been reported that Fe^{++} and phosphate are required respectively for the oxidation and hydrolysis reactions (Suda & Takeda, 1950). The hydrolytic enzyme is said to be the one previously known as acyl-pyruvase (Meister & Greenstein, 1948) and as the triacetic acid hydrolysing enzyme (Connors & Stotz, 1949).

Felix *et al.* (1937) excluded *p*-hydroxyphenylpyruvate and homogentisic acid as intermediates in tyrosine oxidation, although they also found both these compounds to be oxidized by their system. Being unaware of the tyrosine transaminase reaction, and having eliminated the possibility of oxidative deamination of L-tyrosine, they concluded that *p*-hydroxyphenylpyruvate was not formed from tyrosine. The metabolism of tyrosine through homogentisic acid to acetoacetate entirely by oxidative reactions was not considered possible, because this conversion would require five atoms of oxygen instead of the observed four. The transamination, replacing an oxidative deamination, negates this objection to homogentisic acid as an intermediate. The presence of the transaminase, which so alters their conclusions, was actually excluded by them on the apparently good evidence that α-ketoglutarate and tyrosine in a fresh liver homogenate did not form glutamic acid. However, they subsequently found that alanine was formed during tyrosine oxidation (Felix & Zorn, 1941). Lerner (1949) has demonstrated that the carbon skeleton of

alanine formed during tyrosine oxidation does not come from the tyrosine side chain, as Felix & Zorn suggested. These results can be explained by two successive transaminase reactions now known to occur in liver:

(1) L-Tyrosine + α-ketoglutarate → *p*-hydroxyphenylpyruvate + L-glutamate.

(2) L-Glutamate + pyruvate → α-ketoglutarate + L-alanine.

By these reactions tyrosine oxidation can occur in homogenates which contain pyruvate and only catalytic amounts of α-ketoglutarate. The accumulation in this way of alanine instead of glutamate has also been demonstrated chromatographically in the dialysed soluble system.

From the present work, and that of Ravdin & Crandall (1950), the oxidation of L-tyrosine in liver can be finally formulated as follows:

(1) L-Tyrosine + α-ketoglutarate → *p*-hydroxyphenylpyruvate + L-glutamate.

(2) *p*-Hydroxyphenylpyruvate + $\frac{1}{2}O_2$ → 2:5-dihydroxyphenylpyruvate.

(3) 2:5-Dihydroxyphenylpyruvate + $\frac{1}{2}O_2$ → homogentisate + CO_2.

(4) Homogentisate + O_2 → fumaryl-acetoacetate.

(5) Fumaryl-acetoacetate → fumarate + acetoacetate.

This formulation is consistent with the other work quoted, and describes the tyrosine metabolism of at least several species. Interruptions of the appropriate reactions in this pathway can account for the excretion of *p*-hydroxyphenylpyruvate in Medes's case of tyrosinosis (Medes, 1932) and in scorbutic guinea pigs and premature infants, and for the excretion of homogentisic acid by alcaptonurics.

Rienits (1950) and Painter & Zilva (1950) demonstrated definite, but much smaller, effects of ascorbic acid on L-tyrosine oxidation in liver homogenates than the effect which has been demonstrated for the oxidation of *p*-hydroxyphenylpyruvate in the present work. Only a small increase in tyrosine oxidation was observed, because the reaction in homogenates without added α-ketoglutarate was largely limited by the transaminase step. Also much larger amounts of ascorbic acid were necessary to produce an effect, probably due in part to the destruction of added ascorbic acid by the cytochrome system and in part to the fact that even undialysed soluble preparations require more ascorbic acid than do the dialysed ones. In the present study with dialysed preparations no reaction occurred after the first few minutes in the absence of added ascorbic acid, and though the requirement varied, only catalytic amounts of ascorbic acid were needed for long-continued reactions. 'Supersaturated levels' of ascorbic acid were not necessary.

The effect of ascorbic acid on *p*-hydroxyphenylpyruvate oxidation is not produced by other compounds which variously share similar reducing or oxidizing properties (glutathione and peroxide) or a similar reductone structure (dihydroxymaleic acid). Of the compounds tested this quite specific effect is shared only by *iso*ascorbic acid, which also possesses some antiscorbutic activity.

Much greater amounts of ascorbic acid are required in guinea pigs to maintain a normal tyrosine metabolism than are required to prevent scurvy. For this reason it has been stated that the interaction between D-ascorbic acid and L-tyrosine does not indicate a connexion between the normal function of ascorbic acid and the normal metabolism of tyrosine. However, the derangement of the tyrosine metabolism is tested for by feeding abnormally large amounts of tyrosine (0·5 g./day). When less tyrosine is given (0·3 g.) the metabolic derangement is manifested only after greater ascorbic acid depletion (14 days instead of 8) (Painter & Zilva, 1947). When very large doses of tyrosine were given, there were indications that *p*-hydroxyphenylpyruvate was excreted even by animals saturated with ascorbic acid. The amount of ascorbic acid needed to assure the metabolism of tyrosine appears to be related to the amount of tyrosine administered. The lower antiscorbutic levels of ascorbic acid may then suffice for the metabolism of the much smaller physiological amounts of tyrosine and this action in tyrosine metabolism may then be a part of the vitamin function of ascorbic acid. The concentrations of ascorbic acid that were necessary for tyrosine oxidation in the present study were not greater than those which are only antiscorbutic in guinea pigs.

A connexion between these two physiological actions of ascorbic acid also follows from the studies with *iso*ascorbic acid. This compound is antiscorbutic and also acts in tyrosine metabolism in guinea pigs (Sealock & Silberstein, 1940). In both of these actions it is one-twentieth to one-fiftieth as effective as ascorbic acid (Dalmer & Moll, 1933; Demole, 1934). The suggestion has been made that ascorbic and *iso*ascorbic acids are really of equal activity, and that the apparent effect of *iso*ascorbic acid is reduced by its poor retention in the body (Painter & Zilva, 1950). That they do have equal activity, at least in tyrosine oxidation, has been demonstrated in the present *in vitro* system. Similarly, D-glucoascorbic acid is not retained at all in the body and has no antiscorbutic activity (Zilva, 1935), and yet also showed activity in the oxidation of tyrosine by liver homogenates (Painter & Zilva, 1950).

SUMMARY

1. A series of enzymes in the soluble fraction of liver homogenates catalyses the oxidation of L-tyrosine to acetoacetate. In this reaction one

molecule of L-tyrosine is oxidized with uptake of four atoms of oxygen and the formation of one molecule of acetoacetate.

2. Additions of α-ketoglutarate and catalytic amounts of ascorbic acid are required for the activity of the dialysed enzyme.

3. *p*-Hydroxyphenylpyruvate, 2:5-dihydroxyphenylpyruvate, and homogentisic acid are also oxidized to acetoacetate by this system, at rates comparable with that of tyrosine oxidation. *p*-Hydroxyphenylpyruvate and homogentisic acid are formed from tyrosine in the system, and have been identified as intermediates in the reaction.

4. *p*-Hydroxyphenylpyruvate is formed by the first enzyme in the series, an L-tyrosine-glutamic acid transaminase. This step can be made to limit the rate of the overall reaction in the absence of its coenzyme, pyridoxal phosphate. *p*-Hydroxyphenylpyruvate accumulates anaerobically, or, in the absence of ascorbic acid, aerobically.

5. Homogentisic acid is formed by the further oxidation of *p*-hydroxyphenylpyruvate, probably through 2:5-dihydroxyphenylpyruvate as an intermediate. Catalytic amounts of ascorbic acid are required for this reaction. Homogentisic acid can be accumulated in an enzyme mixture treated at pH 5, which destroys the system oxidizing homogentisic acid.

6. Ascorbic and *iso*ascorbic acids are equally effective in promoting the oxidation of *p*-hydroxyphenylpyruvate, and so far could not be replaced by other compounds tested. The possibility that the action in tyrosine metabolism of ascorbic acid is part of its vitamin action is discussed.

REFERENCES

Bernheim, F. & Bernheim, M. L. C. (1934). *J. biol. Chem.* **107**, 275.
Bray, H. G., Thorpe, W. V. & White, K. (1950). *Biochem. J.* **46**, 271.
Cammarata, P. S. & Cohen, P. P. (1950). *J. biol. Chem.* **187**, 439.
Connors, W. M. & Stotz, E. (1949). *J. biol. Chem.* **178**, 881.
Dalmer, O. & Moll, T. (1933). *Hoppe-Seyl. Z.* **123**, 116.
Datta, S. P., Harris, H. & Rees, K. R. (1950). *Biochem. J.* **46**, xxvi.
Demole, V. (1934). *Biochem. J.* **28**, 770.
Edson, N. L. (1935*a*). *Biochem. J.* **29**, 2082.
Edson, N. L. (1935*b*). *Biochem. J.* **29**, 2498.
Feldman, L. E. & Gunsalus, I. C. (1950). *J. biol. Chem.* **187**, 821.
Felix, K. & Zorn, K. (1941). *Hoppe-Seyl. Z.* **268**, 257.
Felix, K., Zorn, K. & Dirr-Kaltenbach, H. (1937). *Hoppe-Seyl. Z.* **277**, 141.
Friedman, E. (1908). *Beitr. chem. Physiol. Path.* **11**, 304.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gilbert, G. A. & Swallow, A. J. (1950). *Biochem. J.* **47**, 506.
Hird, F. J. R. & Rowsell, E. V. (1950). *Nature, Lond.*, **166**, 517.
Knox, W. E. & Mehler, A. (1950). *J. biol. Chem.* **187**, 419.
LeMay-Knox, M. & Knox, W. E. (1951). *Biochem. J.* **48**, xxii.
Lerner, A. B. (1949). *J. biol. Chem.* **181**, 281.
Levine, S. Z., Marples, E. & Gordon, H. H. (1941). *J. clin. Invest.* **20**, 199.
Medes, G. (1932). *Biochem. J.* **27**, 917.
Meister, A. & Greenstein, J. (1948). *J. biol. Chem.* **175**, 573.
Meyer, F. (1901). *Dtsch. Arch. klin. Med.* **70**, 443.
Neubauer, O. (1904). *Hoppe-Seyl. Z.* **42**, 81.
Neubauer, O. (1909). *Dtsch. Arch. klin. Med.* **95**, 211.
Neubauer, O. & Flatow, L. (1907). *Hoppe-Seyl. Z.* **52**, 375.
Neuberger, A. (1947). *Biochem. J.* **41**, 431.
Neuberger, A., Rimington, C. & Wilson, J. M. G. (1947). *Biochem. J.* **41**, 438.
Painter, H. A. & Zilva, S. S. (1947). *Biochem. J.* **41**, 511.
Painter, H. A. & Zilva, S. S. (1950). *Biochem. J.* **46**, 542.
Ravdin, R. G. & Crandall, D. I. (1950). *Fed. Proc.* **9**, 218.
Rienits, K. E. (1950). *J. biol. Chem.* **182**, 11.
Schepartz, B. & Gurin, S. (1949). *J. biol. Chem.* **180**, 663.
Sealock, R. R. & Goodland, R. L. (1949). *J. biol. Chem.* **178**, 939.
Sealock, R. R. & Silberstein, H. E. (1940). *J. biol. Chem.* **135**, 251.
Smith, J. D. (1950). Unpublished observations.
Stumpf, P. K. & Green, D. E. (1944). *J. biol. Chem.* **153**, 387.
Suda, M. & Takeda, Y. (1950). *Med. J. Osaka Univ.* **2**, 41.
Weinhouse, S. & Millington, R. H. (1948). *J. biol. Chem.* **175**, 995.
Woodruff, C. W. & Darby, W. J. (1948). *J. biol. Chem.* **172**, 851.
Zilva, S. S. (1935). *Biochem. J.* **29**, 1612.
Zorn, K. (1940). *Hoppe-Seyl. Z.* **266**, 239.

Some Factors Affecting Anthocyanin Synthesis in Cress Seedlings

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Mapson & Cruickshank (1947) and Mapson, Cruickshank & Chen (1949), in their work on the effects of various sugars and salts upon the accumulation of ascorbic acid in cress seedlings, had noticed that the pinkness of their metaphosphoric acid extracts varied with the various treatments and that, in general, those seedlings containing most ascorbic acid also contained most anthocyanin. The present work describes the quantitative effects on anthocyanin production of feeding seedlings with various sugars and of certain other experimental treatments.

Few attempts have been made in the past to determine whether there is a quantitative relationship between the production of anthocyanin and the quantities of metabolites artificially supplied. Much work has been done, however, on the qualitative aspects of sugar feeding, especially in regard to the effects of different sugars. For reviews of the earlier work reference should be made to Wheldale (1916) and Blank (1947). More recently, Edmondson & Thimann (1947) and Thimann & Edmondson (1949) have shown that in *Spirodela oligorrhiza* the anthocyanin content is increased by feeding with sucrose and fructose, sucrose being the more effective. Glucose was found to have little or no effect.

The question of the necessity or otherwise of light for anthocyanin formation has never been resolved, although Linsbauer (1901), Kuilman (1930) and Karstens (1939) claimed to have shown that a photochemical reaction is necessary for the formation of anthocyanin in *Fagopyrum esculentum*. Edmondson & Thimann (1947) and Thimann & Edmondson (1949) found that pigment production in *Spirodela oligorrhiza* was increased by high light intensity (150–800 foot candles).

METHODS

Conditions of growth. To avoid the effects of variation in temperature and photosynthetic activity, all the experiments were carried out at 20° and, except when the effect of light itself was being investigated, in the dark. The seedlings were grown in white porcelain pots with metal lids, the details of the germination conditions being as follows. The floor of each pot was covered with a filter paper (Whatman no. 1) and 250 seeds (0·7 g.) were added. For the initial watering, 9·0 ml. of glass-distilled water or of a solution of the sugar, containing in each case $HgCl_2$ (50 p.p.m.) to suppress bacterial and fungal growth, were given. This was sufficient for the first 2 days and, subsequently, each pot received 3·0 ml./day for the duration of the experiment.

Method of anthocyanin estimation. The pigment was identified by paper chromatography as chrysanthemin (cyanidin-3-monoside). Daily estimations were made on 200 seedlings from each pot from the 3rd to the 6th days of the experiment and the results are expressed as mg. chrysanthemin per 200 seedlings to avoid complications arising from the effects of the various treatments on the rate of growth. The anthocyanin was extracted by a modification of the method of Karstens (1939). This involved extracting with boiling acid ethanol and transference to distilled water after addition to the filtered extract of 1·6 vol. of a mixture of ether, amyl alcohol and benzene (1:1:2 by vol.).

The resulting solution was made up to 40 ml. with 50% ethanol and the pigment measured colorimetrically using a Spekker photoelectric absorptiometer. All the estimations were done in duplicate, the results plotted being the average of the values obtained. Duplicates differed from each other by less than 20%, the biggest discrepancies being obtained when the more extreme treatments, e.g. feeding with 4% glucose, were used.

Sugar estimations. Total reducing sugars were estimated by the method of Somogyi (1937), as modified by Hanes and described by Hockenhull & Herbert (1945). The reducing values are given in glucose equivalents and have been graphed as mg. per 200 seedlings. Pentoses and other non-fermentable substances were determined by estimating the reducing power after the fermentable sugars had been removed by incubation with yeast. The only hexose sugars present in the material had been shown to be glucose and fructose (Jermyn & Isherwood, 1949), and these were calculated as being equivalent to the total reducing sugars minus the non-fermentable fraction.

Sucrose was estimated by determining the difference in reducing value after hydrolysis with invertase, and fructose was estimated by the method suggested by Roe (1934) and modified by Cole (1942) as follows. A sample was made up to 4 ml. with water and to it were added 6 ml. resorcinol reagent (0·15% in 96% (v/v) ethanol) and 6 ml. HCl-$FeCl_3$ reagent (7·5 mg. $FeCl_3$/100 ml. conc. HCl). The mixture was heated for 30 min. at 75°, together with a blank and two standard fructose solutions treated similarly. The colour developing was estimated in a Spekker photoelectric absorptiometer previously calibrated with standard fructose solutions.

Glucose was taken as being the total fermentable value of the solution less the fructose equivalent.

RESULTS

Effect of sugar feeding on anthocyanin content

Seedlings were grown in the dark on 1 % solutions of the following sugars: glucose, sucrose, fructose, invert sugar, sorbose, galactose and arabinose. Water controls were grown simultaneously. Fig. 1*a* shows the changes in the anthocyanin and sugar contents of the controls, and Figs. 1*b–h* those due to the various sugars. With all the sugars there was a stimulation of anthocyanin production above that in seedlings grown on water alone.

Effect of sugar feeding on sugar content

In cress seedlings grown on water only (Fig. 1*a*) the initial sucrose content of 20 mg./200 seedlings decreased, until by the 6th day it had a value of about 3 mg./200 seedlings. At the same time the total fermentable hexose content rose from zero to a peak at about 96 hr. The hexose is made up of glucose and fructose in roughly constant proportions. There was a slight rise in non-fermentable reducing substances from 13 to about 20 mg./200 seedlings.

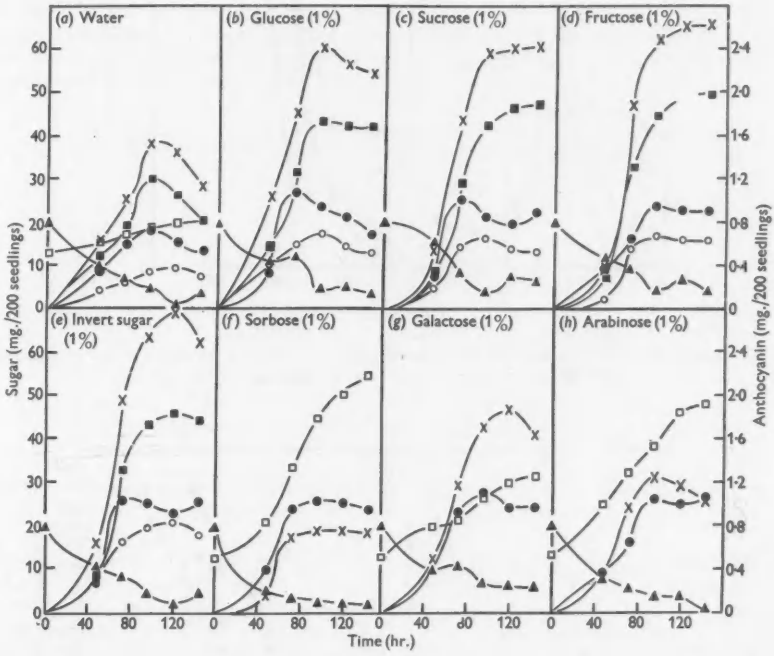

Fig. 1. Changes in the anthocyanin and sugar contents of cress seedlings grown on water and sugars. ●—●, anthocyanin; ×—×, hexose (glucose + fructose); ■—■, glucose; ○—○, fructose; ▲—▲, sucrose; □—□, non-fermentable sugars.

Fig. 2 shows the changes in anthocyanin and sugar contents found on feeding with 2 and 4 % glucose respectively. There was a 150 % stimulation with 2 % glucose, and the curve obtained with 4 % glucose suggests that the peak stimulation might be of the order of 450 %. Fig. 3 shows the relationship between the anthocyanin content and the hexose (glucose + fructose) and total sugar contents. It will be seen that the anthocyanin is more closely related to the latter than to the former.

Feeding 1 % solutions of glucose, sucrose, fructose or invert sugar (Fig. 1*b–e*) caused a slight lag in the disappearance of sucrose and an increase in the total hexose to a value about 23 mg./200 seedlings higher than that in the water-fed seedlings. This was made up of glucose and fructose in approximately constant proportions. Glucose, sucrose and fructose are apparently in equilibrium in the plant, and artificially supplied glucose, sucrose or fructose are interconverted to restore the internal balance.

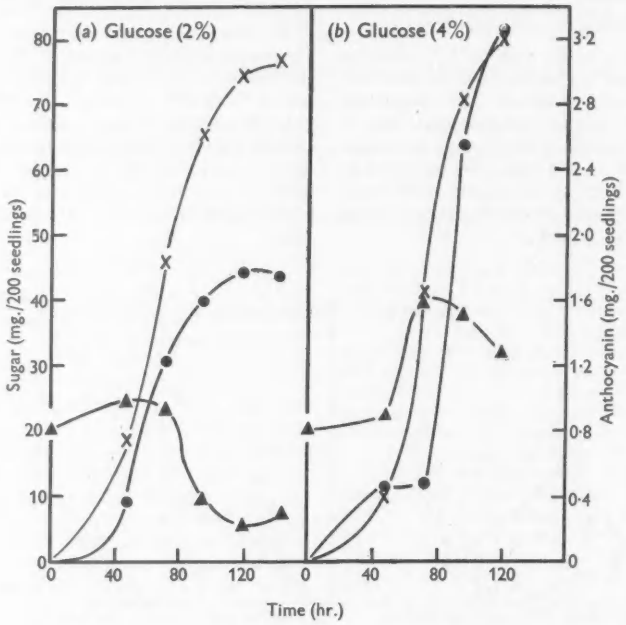

Fig. 2. Changes in the anthocyanin and sugar contents of cress seedlings grown on 2 and 4% glucose. ●—●, anthocyanin; ×—×, hexose; ▲—▲, sucrose.

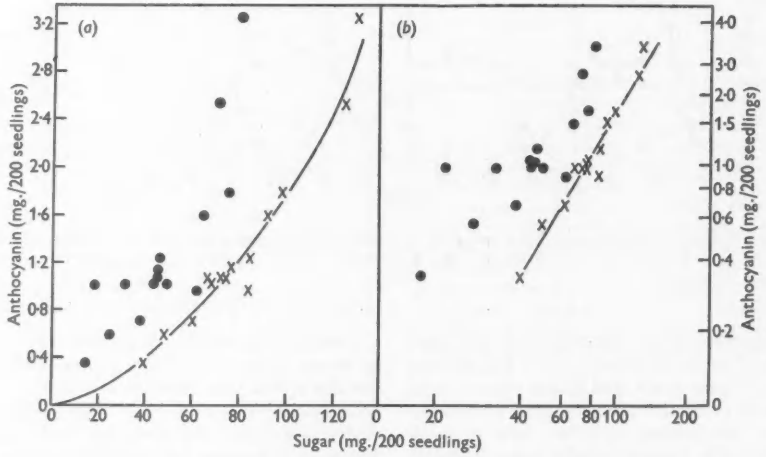

Fig. 3. Relationship between anthocyanin and hexose (●) and anthocyanin and total sugar (×). (*a*) straight plot; (*b*) logarithmic plot.

The additions of 1% sorbose and arabinose (Fig. 1*f*, *h*) appear to accelerate the sucrose breakdown and, especially with sorbose, to depress the production of hexoses, possibly by affecting the rate of fat mobilization. In both cases there was a considerable increase in the non-fermentable fraction, i.e. from about 13 to 50 mg./200 seedlings.

With 1% galactose (Fig. 1*g*) there was a smaller increase in the non-fermentable fraction and also an increase in the fermentable hexoses. Galactose, however, has always been reported to be non-fermentable by yeast, and this fact was checked experimentally. It may be that galactose is converted by the plant into a fermentable hexose or that it is less readily absorbed than other sugars (thus accounting for the lower non-fermentable fraction) and accelerates the mobilization of fat reserves (thus causing an increase in the hexose content).

therefore, germinated in the following solutions, and anthocyanin estimations and sugar analyses carried out to find if there was a similar effect on the pigment production: (i) water; (ii) 0·02 M-ammonium sulphate; (iii) 0·02 M-ammonium sulphate + 1% glucose; (iv) 0·02 M-ammonium sulphate + 2% glucose.

The result for (i) is shown in Fig. 1*a* and for (ii), (iii) and (iv) in Fig. 4*a–c*. The anthocyanin curves for (i), (ii) and (iii) lie so close together that the difference between them is well within the experimental error. The curve for (iv) is similar to that obtained by feeding cress seedlings with 1% glucose in the absence of ammonium sulphate. Although feeding with 0·02 M-ammonium sulphate reduced the internal sugar content to about one-third of its normal value, there was no appreciable reduction in the anthocyanin production. On the other hand, restoration of the content to its normal value by artificial

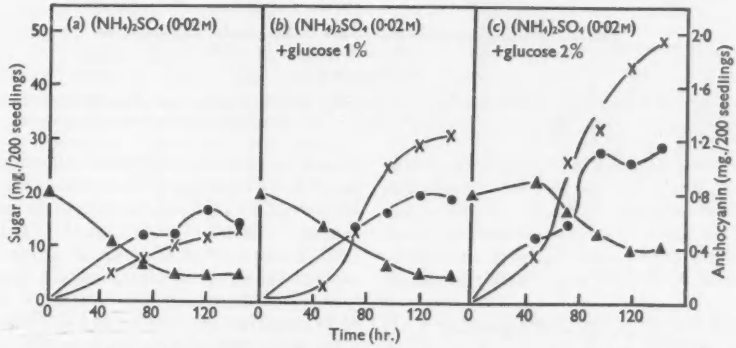

Fig. 4. Changes in the anthocyanin and sugar contents of cress seedlings grown on $(NH_4)_2SO_4$ with and without glucose. ●—●, anthocyanin; ×—×, hexose; ▲—▲, sucrose.

Feeding with either 2 or 4% glucose resulted in an initial synthesis of sucrose—about 5 mg./200 seedlings with 2% and 20 mg. with 4%. The increased synthesis with 4% glucose accounts for the fact that there is very little more free hexose to be found on feeding 4% glucose than on feeding 2%. The extra sucrose is, however, soon broken down and does not appear to bear a direct relationship to the amount of free hexose present. From the figures obtained it is impossible to deduce a threshold value for hexose above which sucrose is synthesized, although it may be that this varied according to the stage of germination of the seedlings.

Effect of ammonium sulphate

Mapson & Cruickshank (1947) and Mapson *et al.* (1949) showed that if cress seeds were germinated on 0·02 M-ammonium sulphate the sugar contents of the seedlings were reduced with a concomitant decrease in the amount of ascorbic acid formed. Seeds were, feeding resulted in no stimulation. Feeding ammonium sulphate with 2% glucose, however, pushed the internal sugar content towards that obtained by feeding 1% glucose without ammonium sulphate and there was a similar increase in the anthocyanin content.

Effect of light

Several workers in the past have claimed to have demonstrated that a photochemical reaction is an essential part of the anthocyanin-producing mechanism. For this reason, and because seedlings grown in the dark are developing under abnormal conditions, the effect of light upon the anthocyanin production of cress seedlings was investigated. The seeds were germinated in white porcelain pots as before, but with glass lids in place of the usual metal ones. For the light source, electric lamps of various wattages were used, either naked or masked, and the intensities were measured in foot-candles

(ft.c.), using an 'Avo' direct reading photoelectric light meter. The effects of 0·5, 1·5, 4 and 15 ft.c. and of 4 ft.c. for 48 hr., followed by darkness, were investigated.

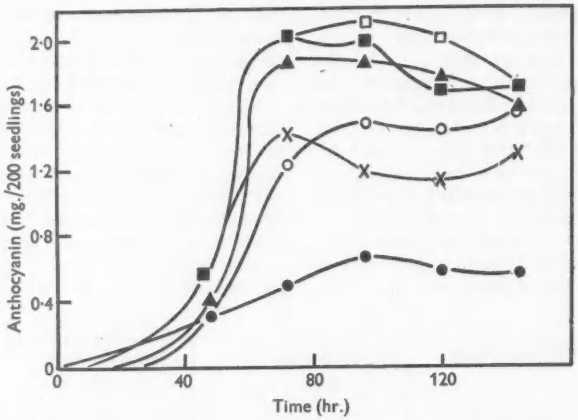

Fig. 5. Effect of light on anthocyanin production by cress seedlings grown on water. ●—●, darkness; ○—○, 0·5 ft.c.; ▲—▲, 1·5 ft.c.; ■—■, 4 ft.c.; □—□, 15 ft.c.; ×—×, 4 ft.c. for 48 hr. and then darkness.

The results are shown in Fig. 5, and in Fig. 6 the chrysanthemin production is plotted against the amount of light supplied in ft.c.hr. Light of an intensity of 0·5 ft.c. resulted in a stimulation of about 130 %, and as the intensity was raised to 15 ft.c. the stimulation rose to 200 %. Transferring seedlings

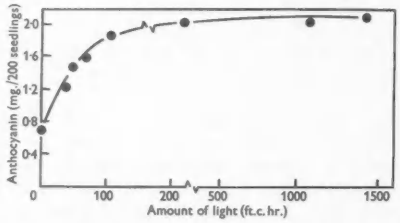

Fig. 6. Relationship between anthocyanin production and amount of light supplied.

which had been grown in the light for 48 hr. into the dark showed that the effect of the light can manifest itself after the source of illumination has been removed. In the 24 hr. after removal to the dark these seedlings produced 0·88 mg. of anthocyanin/200 as compared with control seedlings which had been kept in the dark from the beginning of the experiment and which produced only 0·18 mg. In the same 24 hr., however, those seedlings which had been in the light all the time produced 1·48 mg.

In the case of the cress, we have a system in which a limited amount of anthocyanin is formed even in the dark, and in which the amount of pigment can be increased threefold by an illumination of 15 ft.c. and more than doubled by 0·5 ft.c. That cress seedlings produce a certain amount of anthocyanin in the dark cannot be regarded as very strong evidence against the idea of a photochemical reaction playing a part in anthocyanin formation, since it can be argued that in this case a limited supply of the products of the photochemical reaction may be present in the ungerminated seed. In favour of this idea is the fact that when the seedlings are grown in the light for 48 hr. and then transferred to the dark, most of the total anthocyanin formed is produced after the seedlings have been removed from the light.

DISCUSSION

The results obtained agree with those of the earlier workers in that the anthocyanin content of the seedlings was increased by feeding with glucose, fructose, sucrose and invert sugar. They differ, however, in that the pigment content was also increased by feeding sorbose, galactose and arabinose. The reason for using these latter three sugars was to try to obtain evidence for or against the suggestion which has been put forward that the extra sugar is acting as a substrate for the chain of reactions leading to the production of the anthocyanin molecule, as implied by the hypothesis of Robinson (1935), or whether it upsets the balance of intermediary metabolism, anthocyanin production following as a secondary consequence. If it had been found that only glucose, fructose and sucrose promoted anthocyanin formation, this would have supported the former view. Since, however, such diverse sugars as galactose, sorbose and arabinose

also stimulate anthocyanin production, one must favour the view that the effect of sugar is indirect. Several previous writers (Mirande, 1907; Palladin, 1908; Combes, 1910; Noack, 1922; Blank, 1947) have favoured the latter view.

It is, however, possible that while the glucose, fructose and sucrose feeding was directly supplying a substrate, the feeding of the other sugars was having a different effect. An additional fact in favour of the view that sugar is acting indirectly is that when 4% glucose is fed, an increase of 90 mg. of sugar results in an increase of only 2·9 mg. of anthocyanin.

It is necessary, therefore, to consider the possible effects on general metabolism, especially with regard to growth, of increasing the internal sugar content. Feeding up to 2% sugar had no visible effect on either germination or the rate of growth but, in 4% glucose, elongation of the hypocotyl was retarded, resulting in stunted seedlings. The expansion of the cotyledons appeared to be unaffected, however.

If we now consider the effect of decreasing the internal sugar content by ammonium sulphate feeding, it is necessary to find an explanation why this treatment has comparatively little effect on anthocyanin production. There are two possible lines of explanation. First, it may be that most of the anthocyanin produced in the absence of sugar feeding is formed from pre-existing glycosidic precursors whose conversion into anthocyanin is independent of sugar concentration. The second, and more probable, arises from the fact that the hyperbolic form of the relationship between anthocyanin production and sugar content determines that, at low sugar concentration, large differences are accompanied by only slight variations in anthocyanin content.

Low light intensities can stimulate anthocyanin formation independently of sugar feeding, showing that more pigment can be produced even in the absence of an increased supply of nutrient. All the above considerations tend to support the view that the effect of sugar feeding is stimulatory rather than due to the piling up of substrate for the chain of synthetic reactions. Nevertheless, there is no conclusive proof that sugar is not the immediate substrate for these reactions.

SUMMARY

1. The normal anthocyanin accumulation of cress seedlings grown on water in the dark can be increased by feeding 1% solutions of the following sugars: glucose, fructose, sucrose, invert sugar, galactose, sorbose and arabinose.

2. Whereas 1% glucose causes a stimulation in the anthocyanin production of 50% above normal, 2% glucose causes a stimulation of 150% and 4% glucose a stimulation of 450%. Anthocyanin production is related more closely to total sugar content than to the content of any one sugar.

3. Although feeding with 0·02 M-ammonium sulphate causes a reduction in the internal sugar content to approximately one-third of its normal value, there is no concomitant reduction in anthocyanin content. The possible reason for this is discussed.

4. Low light intensities stimulate the anthocyanin production of cress seedlings grown on water, the curve relating the amount of pigment produced to the amount of light received having the form of a rectangular hyperbola. It has been shown that the effect of the light may manifest itself after the source of the illumination has been removed.

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REFERENCES

Blank, F. (1947). *Bot. Rev.* **13**, 241.
Cole, S. W. (1942). Personal communication.
Combes, R. (1910). *Rev. gén. Bot.* **22**, 177.
Edmondson, Y. H. & Thimann, K. V. (1947). *Amer. J. Bot.* **34**, 598.
Hockenhull, D. J. D. & Herbert, D. (1945). *Biochem. J.* **39**, 102.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Karstens, W. K. H. (1939). *Rec. Trav. bot. néerland.* **36**, 85.
Kuilman, L. W. (1930). *Rec. Trav. bot. néerland.* **27**, 287.
Linsbauer, L. (1901). *Öst. bot. Z.* **51**, 1.
Mapson, L. W. & Cruickshank, E. M. (1947). *Biochem. J.* **41**, 197.
Mapson, L. W., Cruickshank, E. M. & Chen, Y.-T. (1949). *Biochem. J.* **45**, 171.
Mirande, M. (1907). *C.R. Acad. Sci., Paris*, **145**, 1300.
Noack, K. (1922). *Z. Bot.* **14**, 1.
Palladin, W. (1908). *Ber. dtsch. bot. Ges.* **26***a*, 389.
Robinson, R. (1935). *Nature, Lond.*, **135**, 732.
Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Somogyi, M. (1937). *J. biol. Chem.* **117**, 771.
Thimann, K. V. & Edmondson, Y. H. (1949). *Arch. Biochem.* **22**, 33.
Wheldale, M. (1916). *The Anthocyanin Pigments of Plants*, 1st ed. Cambridge University Press.

The Nitration of Benzoic and Phenylacetic Acids and Phenylalanine: the Chemical Basis of the Mohler and Kapeller-Adler Reactions

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While developing a method for the estimation of benzoic acid (Dickens & Pearson, 1951), it was found that the main colour-forming substance responsible for the Mohler (1890) test for benzoic acid was not 3:5-dinitrobenzoic acid, the main nitration product, but another compound formed in small quantity during the nitration as had been previously suggested on indirect evidence by Kapeller-Adler (1932). Quantitative estimation of the intensity of the red colour given on reduction in alkaline solution by the various dinitrobenzoic acids has shown, as reported in the present paper, that in contrast to the weak reaction of 3:5-dinitrobenzoic acid, 2:5-dinitrobenzoic acid gives more than one hundred times as strong a colour test when equimolar amounts of these two substances are directly reduced with hydroxylamine in ammoniacal solution by the method described by Grossfeld (1927). This observation called for a re-investigation of the basis of the Mohler test for benzoic acid, the result of which is reported in this paper.

The hot nitration of phenylalanine was thought by Kapeller-Adler (1932) to yield 3:4-dinitrobenzoic acid via its para nitration and oxidative removal of the side chain giving *p*-nitrobenzoic acid followed by the introduction of a second nitro group in the ortho-position to the first. The basis of this interpretation was the fact that the colour given on alkaline reduction of the nitrated phenylalanine was violet, like that of nitrated *p*-nitrobenzoic acid, and not cherry red as it is in the Mohler reaction for benzoic acid. Block & Bolling (1939), who studied the nitration of phenylalanine in some detail, and slightly modified Kapeller-Adler's quantitative method for the micro-estimation of this substance (see Block, 1938), confirmed that a violet colour was given by phenylalanine in contrast to the red colour given by benzoic acid. They also showed, in agreement with Meisenheimer (1903) and Meisenheimer & Patzig (1906), that a number of aromatic compounds containing two nitro groups in the ortho-position to one another also gave a violet, while those having two nitro groups in a para relationship gave a red colour test. The *m*-dinitro compounds gave no colour or only a very weak one. On this evidence they considered that phenylalanine and phenylacetic acid were nitrated to give first the *p*-nitro compounds which were then further nitrated to the 3:4-dinitro compounds, whereas benzoic acid they suggested, as Kapeller-Adler (1932) had previously thought probable, might form 2:5-dinitrobenzoic acid which could be responsible for the cherry-red colour. They were unable, however, to detect 2:5-dinitrobenzoic acid as a nitration product of either benzoic acid or *m*-nitrobenzoic acid under the conditions employed in this test, but they stated that 2:5-dinitrobenzoic acid was formed by nitration of *o*-nitrobenzoic acid. All these statements are based on the differences of colour (red or violet) given by *p*- and *o*-dinitro substituents on reduction in alkaline solution, and we have thought it necessary to examine quantitatively the amounts of these various substances formed on nitration and where possible to check their formation by isolation.

Block & Bolling (1939) also produce evidence which they consider supports the view that they have isolated from the nitration of L-phenylalanine (*A*) 3:4-dinitrophenylalanine. $\frac{1}{2}C_2H_5OH$, m.p. 155°, decomp. 182°. They also describe the isolation after reduction of the nitration mixture by hydrogen sulphide of: (*B*) 'diaci-3:4-dinitrodihydrophenylalanine', m.p. 182–183°, and (*C*) a further impure substance which may have been '3:4-dinitrodihydrobenzoic acid', softening at 65–70°, decomp. 220–250°. (The nomenclature of these reduced dinitro-compounds is mentioned in the Discussion below.) The colours given with ammonia by *B* and *C*, and by *A* after reduction, were violet. The intensities were (*A*) 'over 160%', (*B*) 140% of the colour given by nitration of an equimolar amount of phenylalanine and subsequent treatment by hydroxylamine and ammonia. Substances *A* and *B* gave no ninhydrin reaction for the α-amino group. The evidence for the position of the nitro groups was again based solely on the colour reaction. The analyses for carbon, hydrogen and nitrogen do not support very convincingly the proposed constitutions of *A* and *B*, and no analysis of *C* is given.

Evidently these observations, useful as they are, leave a number of uncertainties, especially the actual amount of colour-forming substance produced, as well as its chemical nature. Some of these points have been studied by us in more detail and are described below.

EXPERIMENTAL

All m.p.'s are uncorrected.

The preparation of 2:5-dinitrobenzoic acid by hot nitration of o-nitrobenzoic acid. *o*-Nitrobenzoic acid (5 g.), potassium nitrate (10 g.) and conc. H_2SO_4 (100 ml., sp.gr. 1·84) were mixed in a flask, closed with a rubber bung and heated for 1·5 hr. at 100°. The cooled mixture was poured into 200 ml. water, and extracted for 16 hr. with ether. The mixed acids were shaken out from the ethereal extract with about 400 ml. ice-cold N-NaOH. The alkaline solution was acidified by the addition of conc. HNO_3 (5–10 ml.), extracted with ether, and the ether evaporated. The solid residue was dissolved in 40 ml. boiling water, and to the boiling solution a solution of 5 g. lead acetate in 10 ml. water was added. On cooling, a precipitate formed which did not dissolve on re-heating. It was filtered from the hot suspension, boiled with 50 ml. water, again filtered hot and washed on the filter with a further 50 ml. boiling water. The precipitate was dissolved in hot dil. HNO_3 and from the cooled solution the nitro compound separated. Yield 0·67 g. (10% of theoretical), m.p. 174–176°, mixed m.p. 176° with authentic 2:5-dinitrobenzoic acid (see below) of m.p. 177°. After a further crystallization from dil. HCl, it had m.p. 177°, alone or mixed with authentic material. (Found: C, 39·6; H, 2·1, N, 12·8. Calc. for $C_7H_4O_6N_2$: C, 39·6; H, 1·9; N, 13·2%.) Methyl ester, m.p. 94°, mixed m.p. with authentic specimen (see below) 94°. (A specimen of 2:4-dinitrobenzoic acid of m.p. 180° gave mixed m.p. 162°; a specimen of 2:6-dinitrobenzoic acid of m.p. 200° gave mixed m.p. 168° with the above analysis specimen of m.p. 177°.)

The comparison specimens of 2:5-dinitrobenzoic acid, m.p. 177°, and its methyl ester, m.p. 94°, were prepared from a sample of 2:5-dinitrotoluene, kindly supplied by Dr H. H. Hodgson, by dichromate oxidation following the method described in *Organic Syntheses* (Gilman, 1932). (The other dinitrobenzoic acids used were prepared by similar methods.)

The basis of the Mohler reaction. The above evidence shows that under conditions closely similar to those used by Mohler (1890) and Waelsch & Klepetar (1935), for the hot nitration of benzoic acid in their respective colorimetric tests for benzoic acid, *o*-nitrobenzoic acid first formed would be further nitrated to give 2:5-dinitrobenzoic acid. Cold nitration of benzoic acid yields some 20% *o*-nitrobenzoic acid mixed with nearly 80% *m*-nitrobenzoic acid; if the former yields 2:5-dinitrobenzoic acid in the hot solution, the 3:5-dinitrobenzoic acid, previously assumed to be the basis of these authors' colour reaction, will be contaminated with 2:5-dinitrobenzoic acid.

Measurements of colour intensity in the Waelsch & Klepetar test as performed on various nitrobenzoic acids is shown in Table 1. It is evident that 3:5-dinitrobenzoic acid—the main product in the hot nitration of benzoic acid—is a very weak chromogen in this test. The fact proved above, that *o*-nitrobenzoic acid is converted to 2:5-dinitrobenzoic acid under the conditions used by Mohler and by Waelsch & Klepetar, and the very high chromogenic value recorded for this substance in Table 1, make it very probable that the Waelsch & Klepetar reaction is really mainly due to the formation of 2:5-dinitrobenzoic acid. A yield of $100 \times 4{\cdot}7/57$, or 8·2% of this compound from nitration of benzoic acid would be necessary if all the colour were due to it. However, part of the colour development is due to the other nitration products. 3:5-Dinitrobenzoic acid, although it represents about 80% of the nitration product under these conditions, would yield only about 7% of the total colour. On the other hand, the yield of 2:5-dinitrobenzoic acid actually isolated by us (10% of theoretical from *o*-nitrobenzoic acid) would account for 30% of the colour given by benzoic acid, and the isolation from the complex nitration mixture was far from quantitative. The above calculation is based on the formation of 20% of the *o*-nitro compound in the mononitro stage of the hot nitration, 10% of which appears finally as 2:5-dinitrobenzoic acid, or 2% of the amount of benzoic acid taken.

In view of the fact that the true chromogen in the Mohler and Waelsch & Klepetar tests is thus shown to be quantitatively a very minor product, formed to the extent of only a few per cent by hot nitration of benzoic acid, it seems unsuitable as the basis of a satisfactory quantitative reaction, as indeed this test has proved to be in our hands (see Dickens & Pearson, 1951).

Nitration of phenylalanine. DL-Phenylalanine (0·825 g.) was nitrated and subsequently reduced with H_2S as described by Block & Bolling (1939). The brown solid (0·74 g.) which separated from the concentrated butanol extract was

Table 1. *Intensity of colour given in the Waelsch & Klepetar test by benzoic acid* (24·4 *mg.* = 0·2 *mmol.*) *and equivalent amounts of its nitro derivatives*

(Benzoic, *o*-, *m*- and *p*-nitrobenzoic acids were nitrated hot, as in the method of Waelsch & Klepetar (1935), followed by reduction of the mixture with hydroxylamine in ammonia. The other compounds were directly reduced by hydroxylamine in ammonia. The optical density ($E_{1\,cm.}$) of the solution (diluted as required) was measured in a 1 cm. cell with a green filter (transmission 5000–5400 A.) and is calculated for the undiluted solution in each instance.)

Acid	$E_{1\,cm.}$	Colour	Mols. of the acid required to give same colour ($E_{1\,cm.}$) as 1 mol. benzoic acid
Benzoic	4·7	Red	1·00
o-Nitrobenzoic	19·6	Red	0·24
2:5-Dinitrobenzoic	57·0	Red	0·082
m-Nitrobenzoic	1·0	Red	4·7
3:5-Dinitrobenzoic	0·41	Red	11·5
2:3-Dinitrobenzoic	2·03	Violet	2·32
3:4-Dinitrobenzoic	30·9	Violet	0·152
p-Nitrobenzoic	6·6	Greenish → violet	—
2:4-Dinitrobenzoic	0·6	Olive	—
2:6-Dinitrobenzoic	0·2	Olive	—
Styphnic acid (2:4:6-trinitroresorcinol)	—	Bright yellow, no trace of red	—

recrystallized from hot water, m.p. 182–184° (decomp.). Found: (1) dried over $CaCl_2$: C, 41·5; H, 4·0; N, 16·3%; (2) dried *in vacuo* at 60° over P_2O_5: C, 41·9; H, 3·8; N, 16·2%. Dinitrophenylalanine ($C_9H_9O_6N_3$) requires: C, 42·4; H, 3·5; N, 16·5%. 'Dinitrodihydrophenylalanine' ($C_9H_{11}O_6N_3$) requires: C, 42·0; H, 4·3; N, 16·3%. Block & Bolling stated that the m.p. of their crop 2 oven-dried at 110° ('presumably diaci-3:4-dinitrodihydrophenylalanine') was 182–183° and found C, 43·6; H, 4·1; N, 16·2%. Unlike the Block & Bolling compound, neither the crude nor the recrystallized material gave a violet colour with ammonia either alone or in the presence of hydroxylamine.

H_2S was passed into neutral solutions of 3:4- and 2:5-dinitrobenzoic acids under the same conditions as used above, and the excess H_2S was blown off with N_2. No colour was developed with NH_3 alone, but after reduction with hydroxylamine the 3:4-compound gave a violet, and the 2:5-compound a red colour with NH_3. It therefore appears that no dihydro compound was formed by passing H_2S into the neutral solution of these dinitro compounds under the above conditions. There is no evidence other than their colour test to support Block & Bolling's statement that the nitration product, m.p. 182–183°, from phenylalanine is the 3:4-isomer. Presumably the greater part of the nitration product consists of other isomers which, since they give no colour test, may be the 3:5- and 2:4-dinitro derivatives.

Nitration of phenylacetic acid. Borsche (1909) pointed out that earlier workers had reported the m.p. of 2:4-dinitrophenylacetic acid as 160°, whereas by nitration of phenylacetic acid below 60° in mixed H_2SO_4-HNO_3 pure 2:4-dinitrophenylacetic acid, m.p. 179–180° (decomp.), is readily obtained after recrystallization. This observation suggested the simultaneous formation of isomeric dinitro compounds, which we investigated by nitration of phenylacetic acid exactly as described by Borsche (1909). The crude solid which separated as pale-yellow crystals on pouring the crude nitration mixture into ice-cold water had m.p. 179–180° (decomp.), 73% yield. It gave on treatment with hydroxylamine and NH_3 a red colour which rapidly changed to violet, the intensity of the latter colour corresponding to an estimated yield of mixed 3:4- and 2:5-dinitrophenylacetic acids amounting to about 8% of the weight of phenylacetic acid taken, on the assumption that the colour intensities for these apparently unknown dinitro compounds are the same as those of the corresponding dinitrobenzoic acids (see below).

On recrystallizing the above crude product from hot water the m.p. of the pure 2:4-dinitrophenylacetic acid was unchanged (179–180°), but the colour test became progressively weaker with recrystallization and after the third recrystallization was negative, showing that the chromogenic component had been completely eliminated, although little material was lost. (Found: C, 42·6; H, 2·7; N, 12·2. Calc. for $C_8H_6O_6N_2$: C, 42·5; H, 2·7; N, 12·4%.) This evidence shows also that with phenylacetic acid the colour-forming nitration products are present only as very minor components of the nitration mixture, and that the main product, 2:4-dinitrophenylacetic acid, when pure does not give this colour test.

The absorption curve of the reduced nitration products derived from phenylalanine and phenylacetic acid. The nitration of weighed amounts of phenylalanine was performed by heating for 20 min. at 100° with conc. H_2SO_4 containing 20% (w/v) KNO_3, followed by reduction of the ice-cold diluted nitration mixture with hydroxylamine and NH_3 (Block, 1938).

Phenylacetic acid was nitrated at 100° as described by Waelsch & Klepetar (1935), followed by heating for 1 min. with hydroxylamine and NH_3 as in the Waelsch & Klepetar method for estimation of benzoic acid.

The light absorption of the coloured solutions thus obtained was determined in the Hilger Uvispek photoelectric spectrophotometer. The resulting curves are shown in Fig. 1, in which the log extinction coefficients are plotted per μg.mol. of substance taken for nitration.

In Fig. 1 are also included absorption curves for the coloured solutions from 3:4- and 2:5-dinitrobenzoic acids, which were directly reduced in ammoniacal solution as in the Waelsch & Klepetar procedure; in these instances the extinction is given per μg.mol. of the dinitro compound actually taken for reduction. From inspection of Fig. 1 it appears that the chromogenic substances arising from the nitration of both phenylalanine and phenylacetic acid are likely to be mixtures of the respective 3:4- and 2:5-dinitro compounds. In the case of phenylacetic acid the colour which develops is very unstable, changing in type from curve *E* (about 0·5 hr. after the reduction) to curve *D* (1·5 hr.); this is accompanied by an obvious shift of colour from red to violet. The other compounds also gave somewhat unstable colours of which the general characteristics are shown in curves *A*, *B* and *C* of Fig. 1.

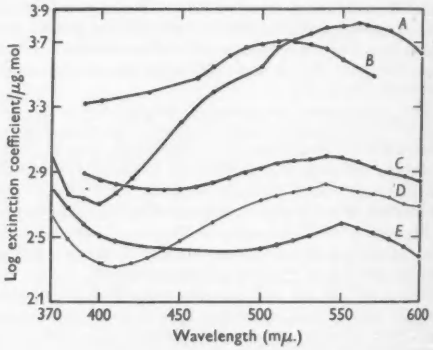


Fig. 1. Absorption spectra of solution obtained on reduction by ammoniacal hydroxylamine of the following: curve *A*, 3:4-dinitrobenzoic acid; curve *B*, 2:5-dinitrobenzoic acid. Curve *C*, nitration and reduction of phenylalanine. Curves *D* and *E*, reduction of crude dinitrophenylacetic acid obtained from phenylacetic acid (see text); curve $E = 0{\cdot}5$ hr., $D = 1{\cdot}5$ hr. after reduction. All extinction coefficients are expressed on the basis of 1 g.mol. of the substance taken for nitration and/or reduction.

As has been mentioned above, the maximum absorption of the phenylalanine compound (at 540 mμ.) lies between those of 3:4- and 2:5-dinitrobenzoic acids (560 and 515 mμ., respectively). If it be assumed that the corresponding dinitro derivatives obtained from phenylalanine and phenylacetic acid give the same molar extinctions as these two dinitrobenzoic acids, the proportion of mixed 3:4- and 2:5-dinitro derivatives arising on nitration are seen to be (Fig. 1) approximately 8 and 15%, respectively, for phenylacetic acid and phenylalanine.

DISCUSSION

This work has shown that complex mixtures of substances are formed in the hot nitration of benzoic acid and phenylalanine under conditions similar to those used for the quantitative estimation of these two substances by the modifications of the Mohler reaction devised by Waelsch & Klepetar (1935) and Kapeller-Adler (1932). These authors' colorimetric reactions depend largely on the formation of substances which are not the main nitration product.

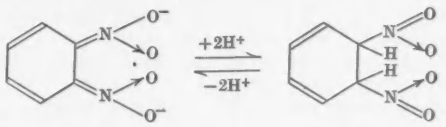
In the case of benzoic acid this was previously suspected to be so. The hot nitration of benzoic acid yields primarily 3:5-dinitrobenzoic acid, which we find contributes less than 8 % of the final (red) colour reaction, obtained on reduction of the dinitro compound in alkaline solution. On the other hand, pure 2:5-dinitrobenzoic acid gives molecule for molecule about 140 times as intense a colour reaction as the 3:5-dinitro compound. We have shown by isolation of the pure compound from the nitration of *o*-nitrobenzoic acid that at least 10 % of 2:5-dinitrobenzoic acid is formed in this reaction, and the losses during this isolation must have been considerable. The formation during the direct nitration of benzoic acid of a 7 % yield of 2:5-dinitrobenzoic acid would be required if this substance accounts for all the colour developed. Since *o*-nitrobenzoic acid on nitration gives about one-third of the colour intensity given by 2:5-dinitrobenzoic acid in this test, it appears that a yield of the 2:5-derivative up to 30 % of theoretical might be possible in this step, although some of this colour may be due to other nitration products. These include 2:3-, 2:4- and 2:6-dinitrobenzoic acids, none of which is a strong colour-former, and also styphnic acid (Griess, 1874) which, however, gives no red colour at all (Table 1). Hence it appears likely that the Mohler reaction is probably mainly due to the formation of 2:5-dinitrobenzoic acid in a yield approximating to 7 % of the benzoic acid taken.

Nitration of phenylacetic acid according to our experiments also yields a mixture of nitro compounds, comprising at least 73 % 2:4-dinitrophenylacetic acid (by isolation), and a mixture, probably of the 2:5- and 3:4-dinitro derivatives, which are together equivalent to about 8 % of the total. The 2:5-compound is probably responsible for the initially formed red colour on reduction of the crude alkaline nitration product, which fairly rapidly passes into a violet colour presumably due to the 3:4-dinitro compound. The pure 2:4-dinitrophenylacetic acid, which is the main product, gives no colour reaction.

These results are of value in considering the behaviour of phenylalanine under the same conditions. As pointed out by Kapeller-Adler (1932) the violet reaction given by this substance is probably due to a 3:4-dinitro compound. This, however, does not constitute a main product, for on attempting the isolation of the reduced dinitro compound prepared as described by Block & Bolling (1939) we obtained as the main crop a substance shown by analysis to be a dinitrophenylalanine which no longer gave this reaction, although it had the same melting point as that given by these authors for the so-called 'diacidihydrodinitro-3:4-phenylalanine'. By analogy with the behaviour of phenylacetic acid it seems probable that the main component may have been 2:4-dinitrophenylalanine.

The absorption spectrum of the crude nitration product from phenylalanine after reduction in ammonia solution resembles that of a mixture of 2:5- and 3:4-dinitrobenzoic acids after the same treatment. This suggests that the chromogenic dinitro compounds produced from phenylalanine have their nitro groups in the same positions. Their total amount is estimated at about 15 % of the total nitration product. Hence, for phenylalanine also, the test depends on the formation of a quantitatively minor product of nitration, and this cannot be regarded as an entirely satisfactory basis for such a micro-estimation.

The nature of the products formed by mild reduction of the *o*- and *p*-dinitrobenzene derivatives has been much studied (cf. Meisenheimer, 1903; Meisenheimer & Patzig, 1906), but their chemical structure remains uncertain. They yield respectively blue-violet and red salts with alkalis which may be formulated as quinonoid structures of the type I. The *m*-dinitro compounds could not form structures of this type, and give no appreciable colour with this test. Block & Bolling (1939) consider that the colourless pseudo-acids from which these coloured salts are derived may be represented as shown at II—the so-called 'diacidihydrodinitro' compound. However, even in the case of the alkali salts of the simpler dinitro aromatic compounds no really satisfactory structure can be considered to be established (Sidgwick, 1942), and we have therefore placed these names in quotation marks in the text.



(I) (II)

SUMMARY

1. The mechanism of the Mohler colour reaction for benzoic acid, and the Kapeller-Adler reaction for phenylalanine, have been investigated by a study of the possible dinitro compounds formed and the colours which they give on reduction with hydroxylamine in alkaline solution.

2. In the case of benzoic acid, 2:5-dinitrobenzoic acid appears to be mainly responsible for the development of the red colour on reduction. The preparation of this substance by nitration of *o*-nitrobenzoic acid is described.

3. The colour formed with phenylacetic acid is considered to be due to a mixture of the 2:5- and 3:4-dinitro compounds.

4. The violet colour given by nitration, followed by alkaline reduction, of phenylalanine appears also to be due to a mixture of products, which probably include 2:5- and 3:4-dinitro derivatives of phenylalanine. The main nitration product was shown by analysis to be a non-chromogenic dinitrophenylalanine.

5. All of the above chromogens are relatively minor products of nitration, and as such do not appear to provide a really satisfactory basis for quantitative estimation of these aromatic substances.

We wish to thank Dr H. H. Hodgson for his kindness in supplying samples of 2:5- and 2:3-dinitrotoluene and Dr O. L. Brady for samples of 2:6- and 3:4-dinitrotoluene, from which the corresponding dinitrobenzoic acids were prepared. All analyses were made by Drs Weiler and Strauss.

REFERENCES

Block, R. J. (1938). *The Determination of the Amino-acids*, p. 75. Minneapolis: Burgess Publishing Co.
Block, R. J. & Bolling, D. (1939). *J. biol. Chem.* **129**, 1.
Borsche, W. (1909). *Ber. dtsch. chem. Ges.* **42**, 1313.
Dickens, F. & Pearson, J. (1951). *Biochem. J.* **48**, 216.
Gilman, H. (1932). *Organic Synth.* Collective Vol. **2**, p. 385.
Griess, P. (1874). *Ber. dtsch. chem. Ges.* **7**, 1225.
Grossfeld, G. (1927). *Z. Untersuch. Nahr.- u. Genussm.* **53**, 467.
Kapeller-Adler, R. (1932). *Biochem. Z.* **252**, 185.
Meisenheimer, J. (1903). *Ber. dtsch. chem. Ges.* **36**, 4174.
Meisenheimer, J. & Patzig, E. (1906). *Ber. dtsch. chem. Ges.* **39**, 2526.
Mohler, M. E. (1890). *Bull. Soc. Chim., Paris*, **3**, 414.
Sidgwick, N. V. (1942). (Revised by Taylor, T. W. J. & Baker, W.) *The Organic Chemistry of Nitrogen*. Oxford: Clarendon Press.
Waelsch, H. & Klepetar, G. (1935). *Hoppe-Seyl. Z.* **236**, 92.

The Action of Sulphydryl Inhibitors upon *iso*Citric Dehydrogenase with Especial Reference to the Behaviour of Some Trivalent Arsenicals*

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The —SH nature of *iso*citric dehydrogenase is still in some doubt because Adler, Euler, Günther & Plass (1939) stated that the enzyme from pig heart was sensitive to iodoacetic acid, whereas Barron & Singer (1945) said that the enzyme from liver was not affected by —SH reagents. It seemed important to examine the effect of arsenical substances on this enzyme in relation to other arsenical studies in this laboratory (Peters, Sinclair & Thompson, 1946). This paper records some observations of the effect of several —SH and other inhibitors upon the enzymes prepared from pig heart and from kidney tissue. A new difference between the action of mono- or di-substituted arsenical compounds has been found, which is thought to be of theoretical interest. Observations upon this enzyme are also related to the recent work in this laboratory (Buffa, Lotspeich, Peters & Wakelin, 1950), in which fluoroacetate was found to inhibit the tricarboxylic acid cycle at the citrate stage.

* A preliminary account of these experiments was given to the XVIIIth International Congress of Physiology, Copenhagen, in August, 1950 (Lotspeich & Peters, 1950).

EXPERIMENTAL

*iso*Citric *dehydrogenase*. The preparation used in most experiments was a dialysed phosphate extract of acetone dried powder of pig heart. In making the acetone powder of pig heart certain modifications of previous methods (Straub, 1942; Ochoa & Weisz-Tabori, 1948) shortened the procedure and increased the enzymic activity of the resulting phosphate extract; the procedure was as follows. A pig heart, obtained as soon as possible after the death of the animal, was immediately freed of fat and gross connective tissue, and cut into pieces which were placed in ice for transport back to the laboratory. All further operations were carried out in the cold room. The pieces of chilled heart muscle were placed in a Waring blender with three times their volume of acetone which had been previously chilled to $-10°$. After blending for 30 sec. the pulp was pressed out through muslin. These procedures were repeated twice more. The resulting heart

pulp was then spread out on a large square of filter paper and allowed to dry in the air, after which it was placed in a vacuum desiccator over $CaCl_2$ until absolutely dry. The fibrous connective tissue was next separated from the fine powder by shaking through muslin. The very fine dry powder which resulted from this sifting process was then used as the starting material for preparation of the phosphate extract, made according to Ochoa (1948), i.e. 1 g. of the powder was extracted successively with 10 ml. and 2 ml. of 0·1 M-phosphate buffer, pH 7·2, at room temperature. After centrifuging, the supernatant fluid was dialysed against running tap water for 6 hr. The precipitate which formed during dialysis was removed by centrifugation. The dialysate was much more active than that described by Ochoa (1948) and had to be diluted tenfold for use; 0·05 ml. of this dilution was used for each test. In some later experiments a similar preparation was made from ox kidney, which had about one-quarter the activity of the heart preparation.

Coenzyme II (*triphosphopyridine nucleotide*). This was prepared from ox liver by a modification of the method of Warburg, Christian & Griese (1935), the details of which were kindly supplied by Dr S. Ochoa. As used the purity was approx. 10% as checked with a sample of Co II of known purity kindly sent by Dr Ochoa.

DL-iso*Citric acid* was prepared in this laboratory by Mr R. W. Wakelin, following the directions of Krebs & Eggleston (1944). The DL form was used in all experiments described; the tables show the calculated amount of D-*iso*citrate present.

Measurement of isocitric *dehydrogenase activity.* The details given by Ochoa (1948) were followed; the activity of the *iso*citric dehydrogenase was measured by the rate of reduction of Co II as observed in the Beckman spectrophotometer at 340 mμ. at room temp. (14·5–18°). In all cases the reaction was started by adding *iso*citrate to the mixture already prepared in the Beckman glass cell, and followed for 10–20 min. Unless specially mentioned all experiments were carried out in Sørenson's 0·05 M-phosphate buffer, pH 7·2, made up from mixtures of KH_2PO_4 and Na_2HPO_4; the final total volume was 3 ml. The amount of Co II preparation used was equivalent to 40 μg. of pure Co II. Using 0·05 ml. of enzyme/3·0 ml., 0·1 ml. 0·2% $MnCl_2.4H_2O$ (0·99 μmol.) was required to produce maximum activity. The optimum pH was found to be between 6·5 and 7·0, confirming Adler *et al.* (1939).

RESULTS

Effect of phosphate and pyrophosphate

Adler *et al.* (1939) stated that inorganic phosphate inhibited the action of the enzyme, and mentioned that it might precipitate the manganese. Fig. 1 shows the effect at pH 7·2 of increasing phosphate concentrations in the presence of a constant amount of Mn. The inhibitory effect produced by phosphate did not appear until approx. 0·1 M-phosphate was reached. Addition of extra manganese removed this inhibition. Grafflin & Ochoa (1950), working at pH 7·4, recommended that phosphate concentrations higher than 0·3 mM should not be used, because of precipitation of manganese phosphate. Evidently, at pH 7·2, the precipitation is much reduced. The inhibitory effect of phosphate, when it arises, is completely explained by its removal of manganese. The inhibition produced by pyrophosphate (Adler *et al.* 1939) was marked at pH 7·2, 3·33 mM producing inhibition with the manganese concentration used; it was confirmed that the addition of more Mn^{++} relieved this inhibition as well.

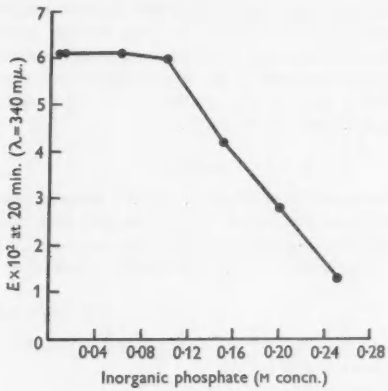


Fig. 1. Effect of inorganic phosphate on *iso*citric dehydrogenase at pH 7·2. Each cell contained phosphate buffer, pH 7·2, of final concentration indicated on the graph; $MnCl_2$ 1·01 μmol.; Co II approx. 0·0819 μmol.; D-*iso*citrate 0·104 μmol.; enzyme extract diluted 1:10, 0·05 ml.; water to final volume of 3 ml.

Relation of Mn^{++} *to activity of* isocitric *dehydrogenase*

On the basis of the observation that the first step in the oxidation of *iso*citrate (eqn. 1) proceeds without added manganese, Ochoa & Weisz-Tabori (1948) considered that manganese was not necessary for this stage, but was needed for the second step, namely decarboxylation of oxalosuccinate (eqn. 2).

$$\text{D-}iso\text{Citrate} + \text{Co II}_{ox.} \rightleftharpoons \text{oxalosuccinate} + \text{Co II}_{red.} \quad (1)$$

$$\text{Oxalosuccinate} \rightleftharpoons \alpha\text{-ketoglutarate} + CO_2. \quad (2)$$

This conclusion would be justified only if the experimental system were completely free of manganese. Accordingly all reagents used in the experiments were tested for manganese. It was found that there were appreciable quantities of manganese in the enzyme used, employing Feigl's (1947) method which uses periodate and 'tetrabase', whereas the Co II (both the specimen used and that from Dr Ochoa) and the *iso*citrate showed none. Since manganese was present, there was no evidence that it was not necessary for the dehydrogenase stage; in fact, it seemed to be essential, because, using the diluted enzyme, practically no activity was found unless manganese was added, whereas with the undiluted enzyme (as used by Ochoa, 1948) there was an appreciable activity.

Effect of substrates of the tricarboxylic acid cycle

DL-iso*Citric acid.* In confirmation of previous work, the enzyme was saturated at 0·02 mM using the amount of Co II stated.

α-Ketoglutarate was slightly inhibitory if present in a concentration of 2 mM acting for 20 min.

Oxalosuccinate and oxaloacetate showed inhibition presumably attributable to the carbon dioxide formed during their spontaneous decarboxylation; all other substrates of the tricarboxylic cycle had no inhibitory effects when tested at a concentration of 1 mg./ml. (approx. 0·2 mM).

Inhibitors

Non-arsenical substances. Table 1 summarizes the results for various —SH inhibitors, including certain metallic compounds other than the arsenicals, which are considered below. The results show that the enzyme is only sensitive to *o*-iodosobenzoate, copper salts and mercury compounds; the inhibition produced by the two metallic compounds was reversible by British anti-lewisite (BAL) and glutathione (Fig. 2). With recrystallized iodoacetic acid no inhibitions were found in concentrations as high as 10^{-3} M; the inhibitory effect observed by Adler *et al.* (1939) was therefore not confirmed. Initially we found that a discoloured preparation of this acid may cause inhibition which disappeared on recrystallization. A typical lachrymator (chloroacetophenone) had no action.

Arsenicals. The experiments with arsenical compounds have given such unexpected results that a short account of the types of the trivalent compounds used is required. In all cases the arsenicals were allowed to act for 15 min. at room temperature before addition of the *iso*citrate. The very insoluble disubstituted compounds were dissolved in 95 % ethanol;

Table 1. *Effect of inhibitors upon* iso*citric dehydrogenase*

(In all cases the inhibitor was allowed to act on the enzyme for 15 min. at room temperature before addition of *iso*citrate. Temperature of test, 15°.)

Inhibitor	Concentration (mM)	Time (min.)	Inhibition (%)
Non-arsenical inhibitors			
Iodine compounds:			
Iodoacetate	1·08	20	0
Iodoacetamide	0·54	20	0
Iodosobenzoate	0·40	20	16
	0·81	20	50
	1·21	20	68
Metallic compounds:			
$CuSO_4$	0·026*	10	0
	0·052	10	50
	0·52†	10	100
p-Chloro-mercuribenzoate	0·02	20	88
p-Chloro-mercuribenzoate + BAL	0·02	20	0
	0·177		
p-Chloro-mercuribenzoate + GSH	0·02	20	21
	2·0		
p-Chloro-mercuribenzoate	0·04	20	100
Phenylmercuric nitrate	0·002	20	19
	0·004	20	77
	0·04	20	100
Other SH inhibitors:			
Sodium maleate	2·08	15	0
Chloroacetophenone	0·65	10	0
$CH_2N(C_2H_3Cl)_2$	1·28	10	0
$(C_2H_3Cl)_3N$	2·85	10	0
Alloxan	4·0	10	0
Other substances:			
p-Aminobenzoate	1·2	20	0
Sodium fluoroacetate	1·66	20	0
Arsenical inhibitors			
Disubstituted arsenicals:			
Diphenylchloroarsine	0·031‡	20	31
	0·063‡	20	100
Other arsenicals:			
Arsenite	1·01	20	0
Lewisite oxide	0·655	20	0

* Rate slowed during first 5 min.
† Effects of Cu were reversed by BAL and GSH (Fig. 2).
‡ For effect of SH compounds, see Fig. 3.

0·1 ml. of this was added to 2·9 ml. of test solution. Controls showed that the amount of ethanol used had no action on the enzyme. Arsenite and the mono-substituted (primary) compound lewisite oxide were studied, and the following disubstituted (secondary) compounds: diphenylchloroarsine (DA), and four dihydrophenarsazines, namely 10-chloro-2-methyl-, 2:8-dichloro-, 10-chloro- and 10-bromo-5:10-dihydrophenarsazines.

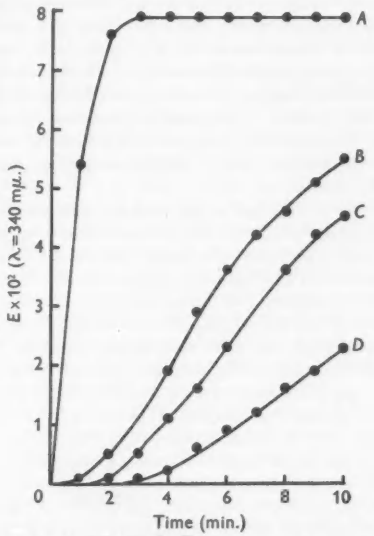


Fig. 2. Inhibition of *iso*citric dehydrogenase by copper and reversal by GSH and BAL. Each cell contained 0·3 ml. 0·1 M-phosphate buffer, pH 7·2; $MnCl_2$, 1·01 μmol.; Co II approximately 0·0819 μmol.; D-*iso*citrate 0·104 μmol.; enzyme extract 0·1 ml. of 1:10 dilution; water to 3 ml. $CuSO_4$ (final concentration 0·052 mM) was allowed to stand with enzyme and other reactants for 15 min. before addition of GSH or BAL and *iso*citrate to start the reaction. Curve *A*, control; curve *B*, copper 0·05 mM, BAL 0·5 mM; curve *C*, copper 0·05 mM, GSH 0·5 mM; curve *D*, copper 0·05 mM.

Contrary to the results with the pyruvate oxidase system which is markedly sensitive to 0·017 mM-lewisite and 0·03 mM-arsenite, the *iso*citric dehydrogenase from pig heart (Table 1) was insensitive to these compounds in a range of concentrations of 0·6–1 mM; it is to be noted that 0·1 mM-As would affect even relatively insensitive enzymes such as succino-dehydrogenase. Any thiol groups present apparently cannot form a stable >As— ring. The preparation from ox kidney was equally insensitive to arsenite, but was found to be slightly inhibited by lewisite oxide at 0·65 mM. This inhibition appeared to differ in some way because it was irreversible and the activity could not be restored by adding BAL. It is difficult to be certain that, when tested at this strength, traces of lewisite II $(ClCH{=}CH)_2AsCl$ were not concerned; these might be present as an impurity.

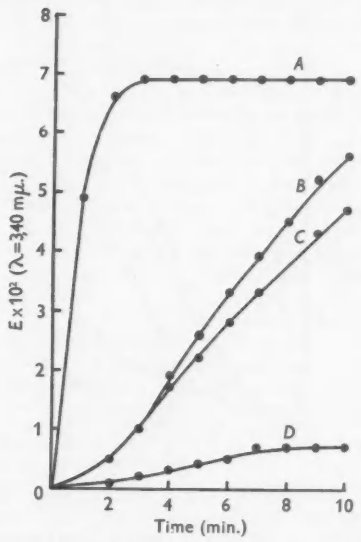
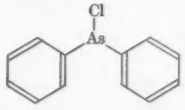
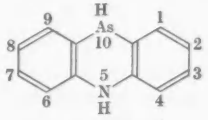


Fig. 3. Inhibition of *iso*citric dehydrogenase by diphenylchloroarsine and reversal by monothiols. All cells contained 0·3 ml. 0·1 M-phosphate buffer, pH 7·2; $MnCl_2$, 1·01 μmol.; Co II approximately 0·0819 μmol.; D-*iso*citrate, 0·104 μmol.; enzyme extract, 0·1 ml. 1:10 dilution; 95% ethanol 0·1 ml.; water to 3 ml. Experimental cells contained diphenylchloroarsine (in 0·1 ml. 95% ethanol) and freshly prepared monothiol to give stated concentrations. Inhibitor stood with enzyme for 15 min. in all experiments. This was also the case before adding monothiol. MTEG = monothioethylene glycol. Curve *A*, control; curve *B*, DA 0·063 mM, GSH 1·3 mM; curve *C*, DA 0·063 mM, MTEG 0·63 mM; curve *D*, DA 0·063 mM.

In strong contrast to the results with the mono-substituted arsenicals, the disubstituted were as toxic as the mercury and copper compounds.



Diphenylchloroarsine



Phenarsazine

Suitable concentrations of BAL and of the mono-thiol compounds, monothioethylene glycol and glutathione, reversed the inhibition (Fig. 3). The interest of this new fact, namely, that there exist —SH groups in one enzyme at least, which can form a stable monothioarsinite, will be discussed below.

This exceptional behaviour of diphenylchloroarsine was explored further, using some other aromatic disubstituted arsenicals, derivatives of phenarsazine.

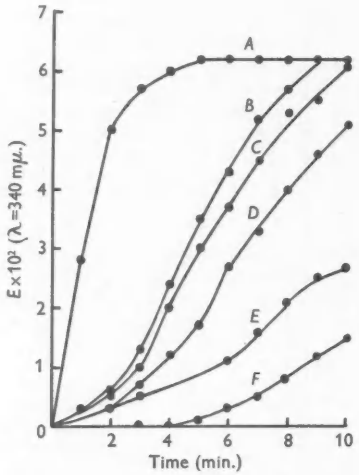


Fig. 4. *iso*Citric dehydrogenase from ox kidney; effect of substituted 5:10-dihydrophenarsazines and comparison with effect of diphenylchloroarsine. Final concentration of arsenicals 0·06 mM. Each cell contained 0·3 ml. 0·1 M-phosphate buffer, pH 7·2; $MnCl_2$, 1·01 μmol.; Co II approx. 0·0819 μmol.; D-*iso*citrate 0·109 μmol., undiluted kidney phosphate extract 0·1 ml.; water to 3 ml. Control cell contained 0·1 ml. 95% ethanol. Experimental cells contained 0·1 ml. 95% ethanol containing inhibitor in sufficient quantity to give final concentration of 0·06 mM. In all cases inhibitor stood at room temperature with reactants for 15 min. before starting reaction with D-*iso*citrate. Curve *A*, control; curve *B*, 10-chloro-2-methyl-5:10-dihydrophenarsazine; curve *C*, 2:8-dichloro-5:10-dihydrophenarsazine; curve *D*, 10-chloro-5:10-dihydrophenarsazine; curve *E*, 10-bromo-5:10-dihydrophenarsazine; curve *F*, diphenylchloroarsine.

Fig. 4 shows that these were all toxic though less so than diphenylchloroarsine.

DISCUSSION

The —SH *problem.* Upon the generally accepted assumption that only —SH groups will respond to these inhibitors, the —SH nature of the *iso*citric dehydrogenase appears to be established by the sensitivity to *o*-iodosobenzoate, to mercury and copper compounds, and to at least one class of arsenicals. The behaviour of the —SH enzymes to the various inhibitors is known to be somewhat capricious, in that the order of activity of inhibitors for a given enzyme is often quite different from the order for another enzyme. This suggests different reactivities of the related —SH groups. It is significant that the —SH group concerned in these *iso*citric dehydrogenase preparations must differ from most of those known at present; considering the order of sensitivity to —SH reagents, it is different in behaviour from those inhibited by lachrymators (Dixon, 1948; Bacq & Desreux, 1946), from myosin (Bailey & Perry, 1947), from triosephosphate dehydrogenase (Dixon, 1937), from succinodehydrogenase (Hopkins & Morgan, 1938) and from the lewisite-sensitive components of the pyruvate oxidase system from brain (Peters *et al.* 1946), which includes the tricarboxylic acid cycle (Peters, 1948), at any rate in part. It is possible that the differences found here between the heart and kidney *iso*citric dehydrogenases might disappear upon further purification.

Relation to the toxicity of arsenical compounds. The work upon lewisite and BAL has cleared up one of the major problems in regard to the biochemical attack made by arsenicals. It has left unsolved one, which is of at least 30 years' standing. McCleland & Peters (1919) found that the toxicity to ciliates of disubstituted trivalent arsenicals was 5–10 times greater than that of the monosubstituted compound; e.g. dimethylchloroarsine > methyldichloroarsine, and diphenylchloroarsine (DA) > phenyl-dichloroarsine; also diphenylarsine oxide was found to be very toxic to urease by Rona & György (1920). The preliminary observations on Protozoa were placed upon an accurate basis for the ciliates, *Colpidium colpoda* and *Glaucoma scintillans*, by Walker (1928) in this laboratory, who also showed a temporary reversal of the toxicity to ciliates of DA by monothioethylene glycol (MTEG) ($HS.CH_2CH_2OH$). It was also early shown that diphenylchloroarsine abolished the nitroprusside reaction for 'fixed' —SH, as well as that given by —SH glutathione; in this respect its action is in line with that of the monosubstituted trivalent arsenicals, as well as arsenite.

In the more recent research in this laboratory unpublished experiments (by Peters & Wakelin) have shown that whereas 0·017 mM-lewisite oxide gave a 50% poisoning of the pyruvate oxidase system from brain under our conditions, DA was without effect in concentrations which it was practicable to try (the substance is relatively insoluble in aqueous solutions). Since this trivalent arsenical is disubstituted it could only form a monothioarsinite and hence could not make a 'dithiol' attack. The question therefore arose as to whether there could exist monothioarsinites formed with DA sufficiently stable to account for the toxicity to ciliates (as also to the irritation caused to the sensitive tissue in the tracheal system in man). If this were true then the non-toxicity to the pyruvate oxidase system could be due to removal of DA by

combination with some other monothiol substances. In pursuance of the idea that thioarsinites with diphenylchloroarsine might be relatively stable owing to the two benzene rings, Peters & Stocken in one unpublished experiment found that the thioarsinite formed by mixing diphenylchloroarsine and a monothiol was relatively stable to the porphyrindin in the test used by Stocken & Thompson (1946). The suggestion, however, lacked biochemical support; the experiments recorded in this paper have now provided this, and have also shown that glutathione can act as an antidote, presumably by forming a more stable monothioarsinite than that of the enzyme-diphenylchloroarsine compound. This leads to the speculation that the animal may be protected systemically against the toxicity of diphenylchloroarsine by the circulating glutathione, in which case compounds like lewisite would be expected to be more successful in penetrating to the tissues owing to the greater instability of their monothiol thioarsinites.

SUMMARY

1. The effect of —SH inhibitors upon *iso*citric dehydrogenase from pig heart has been studied. Some experiments were made with an enzyme prepared from ox kidney.

2. The *iso*citric dehydrogenase preparations needed manganese ions for activity and the inhibitory effect of phosphate was explicable as due to precipitations of manganese.

3. Copper and mercury compounds were inhibitory and the inhibition was reversed by monothiols.

4. With the heart enzyme, the monosubstituted arsenical compounds, arsenite and lewisite oxide, were non-toxic in the concentrations tried; but the disubstituted compounds diphenylchloroarsine and some phenarsazines were toxic. Their toxicity to the enzyme could be reversed by —SH glutathione and by British anti-lewisite (BAL).

5. Maleate, chloroacetophenone, nitrogen mustard, alloxan, iodoacetate and iodoacetamide produced no inhibition, nor did *p*-aminobenzoate and sodium fluoroacetate; some effect was found with iodosobenzoate.

6. With the kidney enzyme, lewisite oxide showed some toxicity not reversible by BAL.

7. Some implications of these facts are discussed; it is concluded that *iso*citric dehydrogenase possesses an —SH grouping of unusual properties.

We are grateful to the late Prof. C. S. Gibson, F.R.S. (Guy's Hospital), for the supply through Prof. R. H. S. Thompson of the phenarsazine compounds used; also to Dr L. A. Stocken for advice.

REFERENCES

Adler, E., von Euler, H., Günther, G. & Plass, M. (1939). *Biochem. J.* **33**, 1028.
Bacq, Z. M. & Desreux, V. (1946). *Acta biol. Belg.* **3**, 369.
Bailey, K. & Perry, S. V. (1947). *Biochem. J.* **41**, xxii.
Barron, E. S. G. & Singer, T. P. (1945). *J. biol. Chem.* **157**, 221.
Buffa, P., Lotspeich, W. D., Peters, R. A. & Wakelin, R. W. (1950). *Biochem. J.* **47**, xvii.
Dixon, M. (1937). *Nature, Lond.*, **140**, 806.
Dixon, M. (1948). *Biochem. Soc. Symp.* **2**, 42.
Feigl, F. (1947). *Qualitative Analysis by Spot Tests*, p. 134. 3rd English ed. trans. by R. E. Olsper. N.Y., Amsterdam: Elsevier Publ. Co. Inc.
Grafflin, A. L. & Ochoa, S. (1950). *Biochim. Biophys. Acta*, **4**, 205.
Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
Lotspeich, W. D. & Peters, R. A. (1950). *Int. physiol. Congr. Abstr.* p. 341.
McCleland, N. & Peters, R. A. (1919). *J. Physiol.* **53**, xv *P.*
Ochoa, S. (1948). *J. biol. Chem.* **174**, 115. And personal communication.
Ochoa, S. & Weisz-Tabori, E. (1948). *J. biol. Chem.* **174**, 123.
Peters, R. A. (1948). *Proc. R. Soc. Med.* **41**, 781.
Peters, R. A., Sinclair, H. M. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 516.
Rona, P. & György, P. (1920). *Biochem. Z.* **111**, 115.
Stocken, L. A. & Thompson, R. H. S. (1946). *Biochem. J.* **40**, 535.
Straub, F. B. (1942). *Hoppe-Seyl. Z.* **275**, 63.
Walker, E. (1928). *Biochem. J.* **22**, 292.
Warburg, O., Christian, W. & Griese, W. (1935). *Biochem. Z.* **282**, 157.

The Biological Action of Substances Related to Thyroxine

2. FURTHER DERIVATIVES OF 3:5-DIIODO-4-HYDROXYBENZOIC ACID

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In Part 1 of this series of communications (Sheahan, Wilkinson & Maclagan, 1951) it was shown that 3:5-diiodo-4-hydroxybenzoic acid exerted a slight antithyroxine activity when tested in mice by the oxygen-consumption method (Maclagan & Sheahan, 1950). Esterification produced a considerable enhancement of the activity, which was maximal when the *n*-butyl ester was used. The present paper describes the study of the effects produced by variation in molecular structure. All the derivatives tested were of the type

which has been shown to be important for antithyroxine potency. They include a number of ethers and acyl derivatives of the hydroxy acid, and, of these, Frieden & Winzler (1949) have reported that the benzyl ether exhibited a tyroxine-inhibitory action in tadpoles, an effect we also observed in mice (Maclagan, Sheahan & Wilkinson, 1949). In addition a number of branched-chain esters of the hydroxy acid were examined.

Wheeler & Liddle (1910) described the preparation of the methyl ester of 3:5-diiodo-4-methoxybenzoic acid by treatment of the hydroxy acid with methyl iodide and alkali, but in our experience poor yields were obtained. Similar results were obtained when 3:5-diiodo-4-hydroxybenzonitrile was methylated by this procedure. Recourse was therefore made to the method of Kalb, Schweizer, Zellner & Barthold (1926), who methylated 2:6-diiodo-4-nitrophenol with excess methyl sulphate in the presence of alkali. By this means the methoxy nitrile and the methyl ester of the methoxy acid were obtained in excellent yields from the hydroxy nitrile and hydroxy acid, respectively. The method was also applicable to the methylation of various alkyl 3:5-diiodo-4-hydroxybenzoates.

The *n*-propyl ether was obtained in moderate yield by treatment of 3:5-diiodo-4-hydroxybenzonitrile with *n*-propyl bromide and alkali, but in addition 3:5-diiodo-4-*n*-propoxybenzamide was obtained from the mother liquors.

Ethyl 3:5-diiodo-4-hydroxybenzoate reacted with benzyl chloride in the presence of alkali to give benzyl ether, but the *n*-propyl ester under similar conditions underwent de-iodination and *n*-propyl 3:5-dihydroxy-4-benzyloxybenzoate was isolated from the reaction mixture.

The branched-chain alkyl esters of 3:5-diiodo-4-hydroxybenzoic acid were prepared by methods similar to that described in our earlier paper (Sheahan *et al.* 1951).

EXPERIMENTAL

Chemical methods

Analyses by Drs Weiler and Strauss, Oxford, and by Herd and Mundy, London. Melting points are uncorrected.

3:5-Diiodo-4-methoxybenzonitrile. 3:5-Diiodo-4-hydroxybenzonitrile (Auwers & Reis, 1896) (18·5 g., 0·05 g.mol.) was dissolved in methanol (100 ml.) and treated with 10 N-NaOH (10 ml.) and methyl iodide (28 g., 0·2 g.mol.). The mixture was heated under reflux for 90 min. While still hot, the precipitated NaI was removed by filtration, and the filtrate cooled and diluted with water (100 ml.). On standing the *product* (2·0 g.) separated as a white precipitate, which crystallized from methanol in colourless needles, m.p. 148°. (Found: C, 25·1; H, 1·7; I, 66·2. $C_8H_5ONI_2$ requires C, 24·9; H, 1·3; I, 66·0%.) No improvement in the yield was effected by varying the proportions of the reactants. The mother liquors yielded 16 g. of unreacted 3:5-diiodo-4-hydroxybenzonitrile, m.p. 202–204°, on acidification.

The methoxy nitrile was, however, obtained in 98% yield by the gradual addition, with vigorous shaking, of 20% NaOH to a hot solution of 3:5-diiodo-4-hydroxybenzonitrile (18·55 g.) in dimethyl sulphate (60 ml.) until the mixture was alkaline to phenolphthalein. The solid (18·7 g.) was then collected, washed with water and dried at 100°. After crystallization from methanol it had m.p. 148°, and proved to be identical with the 3:5-diiodo-4-methoxybenzonitrile described above.

On hydrolysis with NaOH the methoxy nitrile gave 3:5-diiodo-4-methoxybenzoic acid, m.p. 261–262°. Wheeler & Liddle (1910) report m.p. 265° for the acid obtained by hydrolysis of the methyl ester.

Methyl 3:5-diiodo-4-methoxybenzoate was obtained by the gradual addition with vigorous shaking of 20% NaOH to a suspension of 3:5-diiodo-4-hydroxybenzoic acid (10 g.) in dimethyl sulphate (60 ml.) previously heated to 70°. When alkaline to phenolphthalein the solid (9·77 g.) was filtered from the cooled mixture, washed with water and

dried at 20° over silica gel. After recrystallization from methanol it had m.p. 94–95°. Wheeler & Liddle (1910) report m.p. 95°.

Ethyl 3:5-*diiodo*-4-*methoxybenzoate*. 3:5-Diiodo-4-methoxybenzoic acid (1·24 g.) was heated under reflux with ethanol (25 ml.) containing H_2SO_4 (3 drops) for 3 hr. The solution was concentrated to about 10 ml. and the residue poured into a mixture of ice and 2 N-Na_2CO_3 (20 ml.). The solid (1·03 g.) which separated was washed with water and dried over $CaCl_2$. The *ethyl ester* crystallized from light petroleum (b.p. 60–80°) in colourless needles, m.p. 95–96°. (Found: C, 28·3; H, 2·6; I, 59·0. $C_{10}H_{10}O_3I_2$ requires C, 27·8; H, 2·6; I, 58·9%.)

n-*Propyl* 3:5-*diiodo*-4-*methoxybenzoate* was prepared by the method used for the methyl ester from *n*-propyl 3:5-diiodo-4-hydroxybenzoate (8·64 g.) and dimethyl sulphate (30 ml.). It separated as an oil (8·9 g.) which crystallized on storage. Recrystallization from light petroleum (b.p. 40–60°) gave the required *ester* as colourless needles, m.p. 58–59°. (Found: C, 29·8; H, 2·6; I, 56·8. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

The n-*butyl ester* was prepared similarly from *n*-butyl 3:5-diiodo-4-hydroxybenzoate in almost theoretical yield. The oil which separated was induced to crystallize by cooling in ice. It crystallized from light petroleum (b.p. 40–60°) in large colourless prisms, m.p. 45°. (Found: C, 31·4; H, 2·8; I, 55·2. $C_{12}H_{14}O_3I_2$ requires C, 31·3; H, 3·05; I, 55·2%.)

Methyl 3:5-*diiodo*-4-*ethoxybenzoate* was prepared from 3:5-diiodo-4-ethoxybenzoic acid (Wilkinson, 1951) (1·5 g.), methanol (30 ml.) and H_2SO_4 (3 drops) by the method used for ethyl 3:5-diiodo-4-methoxybenzoate. The *product* crystallized from light petroleum (b.p. 80–100°) in colourless needles, m.p. 93°. (Found: C, 28·0; H, 2·2; I, 59·2. $C_{10}H_{10}O_3I_2$ requires C, 27·8; H, 2·3; I, 58·9%.)

The *ethyl ester*, prepared similarly, separated from light petroleum (b.p. 60–80°) in colourless needles, m.p. 71°. (Found: C, 30·3; H, 2·7; I, 57·0. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·1%.)

n-*Propyl* 3:5-*diiodo*-4-*ethoxybenzoate*. *n*-Propyl 3:5-diiodo-4-hydroxybenzoate (8·64 g.) was dissolved in ethyl sulphate (50 ml.) and heated on a steam bath. 20% NaOH (85 ml.) was added gradually to the heated mixture with vigorous shaking over 1·5 hr. The mixture was cooled and diluted with water. The oil, which separated, was induced to solidify on cooling with ice. The *ester* (9·1 g.) was collected, washed with water, and dried at 20° over $CaCl_2$. It crystallized from light petroleum (b.p. 40–60°) in colourless prisms, m.p. 38°. (Found: C, 31·6; H, 3·3; I, 54·5. $C_{12}H_{14}O_3I_2$ requires C, 31·3; H, 3·05; I, 55·2%.)

3:5-*Diiodo*-4-n-*propoxybenzonitrile*. 3:5-Diiodo-4-hydroxybenzonitrile (9·3 g., 0·025 g.mol.) was dissolved in *n*-propanol (50 ml.) and 10 N-NaOH (5 ml.). *n*-Propyl bromide (12·3 g., 0·1 g.mol.) was added and the mixture heated under reflux for 2 hr. After concentration to about 25 ml., the mixture was filtered and diluted with water (10 ml.). The solid (2·07 g.) which separated crystallized from 95% methanol in colourless prisms, m.p. 115°, and proved to consist of the required *nitrile*. (Found: C, 29·1; H, 2·4; I, 62·5. $C_{10}H_9ONI_2$ requires C, 29·1; H, 2·2; I, 61·5%.) Further addition of water precipitated a solid (1·3 g.), m.p. 165–167°, which crystallized from methanol in colourless needles, m.p. 170–171°, identified as 3:5-*diiodo*-4-n-*propoxybenzamide*. (Found: C, 28·2; H, 2·3; N, 3·2; I, 59·0. $C_{10}H_{11}O_2NI_2$ requires C, 27·9; H, 2·5; N, 3·25; I, 59·0%.) Acidification of the mother liquors regenerated unreacted hydroxybenzonitrile (5·4 g.), m.p. 202–205°.

Improved yields of the *n*-propoxyamide were obtained by using twice the above amounts of 10 N-NaOH and *n*-propyl bromide, and prolonging the period of heating to 2·5 hr. By this means the yield of amide was increased to 5·4 g. (50%) and 4·56 g. of unreacted starting material were recovered.

3:5-*Diiodo*-4-n-*propoxybenzoic acid*. The nitrile (2·5 g.) dissolved in hot ethanol (10 ml.) was heated under reflux with 5 N-NaOH (25 ml.) for 2 hr. The solution was diluted with water (10 ml.) and filtered. The filtrate was acidified with HCl to precipitate the *acid* (2·32 g.), which crystallized from benzene in colourless needles, m.p. 191°. (Found: C, 28·1; H, 2·4; I, 58·7. $C_{10}H_{10}O_3I_2$ requires C, 27·8; H, 2·3; I, 58·9%.) The acid was also obtained in good yield by similar treatment of the amide.

The *methyl ester* crystallized from light petroleum (b.p. 40–60°) in colourless needles, m.p. 55°. (Found: C, 29·9; H, 2·65; I, 56·7. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

The *ethyl ester* separated from the same solvent in colourless plates, m.p. 44–45°. (Found: C, 31·6; H, 3·0; I, 55·3. $C_{12}H_{14}O_3I_2$ requires C, 31·3; H, 3·0; I, 55·2%.)

Methyl 3:5-*diiodo*-4-*benzyloxybenzoate*, prepared by esterification of 3:5-diiodo-4-benzyloxybenzoic acid (Frieden & Winzler, 1949), crystallized from methanol in colourless needles, m.p. 123°. (Found: C, 36·5; H, 2·2; I, 51·6. $C_{15}H_{12}O_3I_2$ requires C, 36·5; H, 2·4; I, 51·4%.)

Ethyl 3:5-*diiodo*-4-*benzyloxybenzoate*. Ethyl 3:5-diiodo-4-hydroxybenzoate (Part I) (4·18 g.) in ethanol (15 ml.) was treated with 2 N-NaOH (5 ml.) and benzyl chloride (1·3 g.), and the mixture heated under reflux for 15 min., during which time an oil separated. After cooling, water (10 ml.) was added to complete the separation of the oily product. On refrigeration this was obtained as a solid (1·84 g.). The *ester* was obtained as colourless needles, m.p. 72°, from ethanol. (Found: C, 37·8; H, 2·7; I, 50·2. $C_{16}H_{14}O_3I_2$ requires C, 37·8; H, 2·75; I, 50·0%.)

Attempted preparation of n-*propyl* 3:5-*diiodo*-4-*benzyloxybenzoate*. *n*-Propyl 3:5-diiodo-4-hydroxybenzoate (4·32 g.) in *n*-propanol (15 ml.) was treated as described for the ethyl ester. After removal of the solvent, water was added to precipitate the product, which crystallized from light petroleum (b.p. 60–80°) in colourless clusters, m.p. 98–100°, which did not contain iodine. They appeared to consist of n-*propyl* 3:5-*dihydroxy*-4-*benzyloxybenzoate*. (Found: C, 67·6; H, 6·8. $C_{17}H_{18}O_5$ requires C, 67·6; H, 6·0%.)

The following acyl derivatives of *n*-butyl 3:5-diiodo-4-hydroxybenzoate were prepared by dissolving the ester (2 g.) in pyridine (2 ml.) and adding the acid anhydride or chloride (0·5–1 g.). After standing at room temperature for 30 min., the mixture was heated to 60° for a further 10 min. The cooled mixture was poured into water (20 ml.) and when the gum which separated had solidified it was collected by filtration and washed with water.

Acetate. Highly refractive colourless hexagonal plates, m.p. 80°, from methanol. (Found: C, 32·5; H, 2·9; I, 51·8. $C_{13}H_{14}O_4I_2$ requires C, 32·0; H, 2·9; I, 52·1%.)

Propionate. Large colourless prisms, m.p. 84–85°, from light petroleum (b.p. 40–60°). (Found: C, 34·0; H, 3·15; I, 51·6. $C_{14}H_{16}O_4I_2$ requires C, 34·2; H, 3·3; I, 51·7%.)

Benzoate. Colourless irregular clusters, m.p. 74°, from light petroleum (b.p. 40–60°). (Found: C, 39·5; H, 3·2; I, 45·7. $C_{18}H_{16}O_4I_2$ requires C, 39·3; H, 2·9; I, 46·2%.)

Carbethoxy derivative. Prepared similarly, using ethyl chloroformate (1 ml.). Colourless plates, m.p. 79°, from methanol. (Found: C, 32·5; H, 3·1; I, 48·9. $C_{14}H_{16}O_5I_2$ requires C, 32·5; H, 3·1; I, 49·1%.)

Methyl 3:5-*diiodo*-4-*acetoxybenzoate* was prepared in a similar manner from methyl 3:5-diiodo-4-hydroxybenzoate. It crystallized from methanol in colourless plates, m.p. 121°. (Found: C, 27·1; H, 1·9; I, 57·0. $C_{10}H_8O_4I_2$ requires C, 26·9; H, 1·8; I, 57·0%.)

2-*Ethylbutyl* p-*hydroxybenzoate.* *p*-Hydroxybenzoic acid (27·6 g.) was heated under reflux with 2-ethylbutanol (100 ml.), benzene (100 ml.) and H_2SO_4 (4 g.) for 3 hr., a McIntyre stillhead being employed to separate the water liberated during the reaction. The cooled mixture was neutralized with NaOH, and acidified with acetic acid. The solvents were removed by steam distillation and the residue extracted with ether. The extract was dried (Na_2SO_4), the solvent removed, and the *product* (26·5 g.) distilled. It was obtained as a pale-yellow oil, b.p. 141–142°/0·3 mm. (Found: C, 69·8; H, 8·2. $C_{13}H_{18}O_3$ requires C, 70·2; H, 8·1%.)

Branched-chain alkyl 3:5-*diiodo*-4-*hydroxybenzoates.* The alkyl *p*-hydroxybenzoates were treated with iodine (2 g.mol.) and potassium iodate ($\frac{1}{2}$ g.mol.) in aqueous ethanol as described by Sheahan *et al.* (1951). By this means the following esters were prepared:

iso*Butyl* 3:5-*diiodo*-4-*hydroxybenzoate*, which crystallized from ethanol in colourless needles, m.p. 149°. (Found: C, 29·3; H, 2·7; I, 56·3. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

sec.-*Butyl* 3:5-*diiodo*-4-*hydroxybenzoate.* Colourless plates from 90% ethanol, m.p. 111°. (Found: C, 29·0; H, 2·8; I, 56·2. $C_{11}H_{12}O_3I_2$ requires C, 29·6; H, 2·7; I, 57·0%.)

iso*Amyl* 3:5-*diiodo*-4-*hydroxybenzoate.* Colourless needles from aqueous methanol, m.p. 98–100°. (Found: C, 30·6; H, 3·1; I, 55·2. $C_{12}H_{14}O_3I_2$ requires C, 31·3; H, 3·1; I, 55·2%.)

2-*Ethylbutyl* 3:5-*diiodo*-4-*hydroxybenzoate.* Colourless plates, m.p. 69–70°, from light petroleum (b.p. 60–80°). (Found: C, 33·0; H, 3·5; I, 52·5. $C_{13}H_{16}O_3I_2$ requires C, 32·9; H, 3·5; I, 53·6%.)

3:5-Diiodo-4-phenoxybenzoic acid and its methyl and *n*-butyl esters were provided through the courtesy of Glaxo Laboratories Ltd.

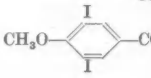
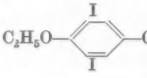
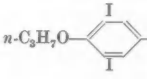
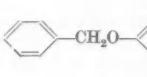
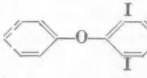
Methods of testing

The compounds were tested for antithyroxine activity in mice by the oxygen-consumption method of Maclagan & Sheahan (1950) with the improvements described by Sheahan *et al.* (1951).

RESULTS

The effects of ether formation on the antithyroxine properties of 3:5-diiodo-4-hydroxybenzoic acid are shown in Fig. 1. The methyl, ethyl and *n*-propyl ethers were slightly more active than the hydroxy acid and equalled the activity of the benzyl ether. The phenyl ether, however, proved the most active of the series, producing a significant reduction of the thyroxine effect at a total dose of 100 mg./kg.

Table 1. *The antithyroxine effects of esters of* 3:5-*diiodo*-4-*alkoxybenzoic acids*

Compound		Total dosage (mg./kg.) which produced: Significant response*	No response
	R = H	400 (69%)	200
	CH_3	—	400
	C_2H_5	100 (48%)	50
	n-C_3H_7	—	400
	n-C_4H_9	—	400
	R = H	400 (77%)	200
	CH_3	400 (25%)	200
	C_2H_5	50 (32%)	25
	n-C_3H_7	—	400
	R = H	400 (53%)	200
	CH_3	1000 (32%)	400
	C_2H_5	—	1000
	R = H	400 (45%)	200
	CH_3	1000 (64%)	400
	C_2H_5	1000 (68%)	400
	R = H	100 (26%)	50
	CH_3	—	400
	n-C_4H_9	—	400

* The figures in brackets indicate the percentage inhibition of the thyroxine effect at the stated dose. The figures were subjected to statistical analysis by 'Student's' *t* test.

Table 1 shows the results obtained when esters of the alkoxy acids were tested. They suggest that the optimal length of hydrocarbon chain associated with

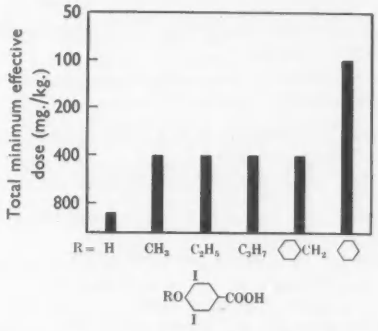
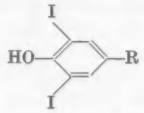


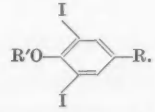
Fig. 1. Antithyroxine effect of a series of ethers of 3:5-diiodo-4-hydroxybenzoic acid.

3:5-diiodo-4-hydroxybenzoic acid is from three to five carbon atoms, which may be in one chain as in the *n*-butyl ester, or in more than one, as in ethyl 3:5-diiodo-4-ethoxybenzoate. This suggests that in this series, a partition effect is involved in the mechanism of the antithyroxine action, but we have not yet completed this aspect of our study. In the case of the methoxy and ethoxy acids, a maximum antithyroxine effect was observed with the ethyl ester, but the esters of the *n*-propoxy, phenoxy and benzyloxy acids were less active than the free acids.

It is noteworthy that in no case was the activity of the alkoxy esters comparable with that of the hydroxy benzoates. It would therefore appear that, assuming some competitive antagonism is exerted against thyroxine, the group



is more effective than



Some acyl derivatives of the active alkyl diiodo-4-hydroxybenzoates were also tested. A number of *O*-acyl substituents in the *n*-butyl ester caused a marked diminution in activity (Fig. 2) whilst the *O*-acetyl derivative of the methyl ester was quite inactive at 400 mg./kg.

A number of branched-chain esters of 3:5-diiodo-4-hydroxybenzoic acid, related to the highly active *n*-butyl ester, were also examined, but their activities were much lower than the isomeric straight-chain compounds (Fig. 3).

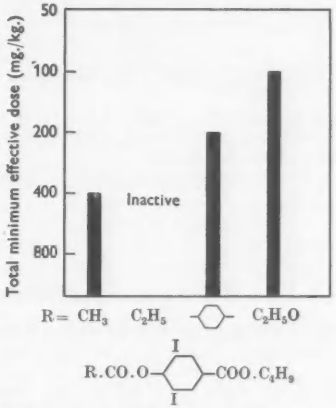


Fig. 2. Antithyroxine effect of acyl derivatives of *n*-butyl 3:5-diiodo-4-hydroxybenzoate.

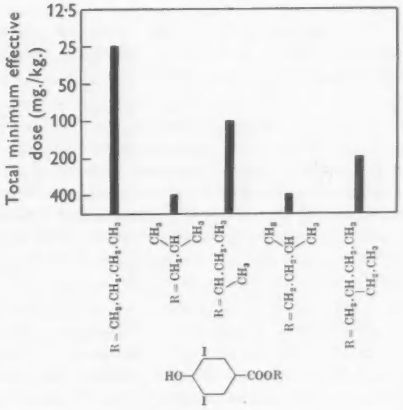


Fig. 3. Antithyroxine effect of branched-chain esters of 3:5-diiodo-4-hydroxybenzoic acid.

3:5-Diiodo-4-methoxybenzonitrile produced a significant reduction of the thyroxine effect at a total dose of 1000 mg./kg., but was inactive at 400 mg./kg. 3:5-Diiodo-4-*n*-propoxybenzonitrile and the corresponding benzamide, however, were inactive at 1000 and 400 mg./kg. respectively.

SUMMARY

1. A series of fifteen 3:5-diiodo-4-alkoxybenzoic acids and their esters has been tested for antithyroxine activity by the oxygen consumption method in mice, but all were markedly inferior to *n*-butyl 3:5-diiodo-4-hydroxybenzoate.

2. Four branched-chain alkyl esters of 3:5-diiodo-4-hydroxybenzoic acid were found to be much less active than the straight-chain esters.

3. 3:5-Diiodo-4-methoxybenzonitrile exerted slight antithyroxine activity, but 3:5-diiodo-4-*n*-propoxybenzonitrile and the corresponding benzamide were inactive.

REFERENCES

Auwers, K. & Reis, J. (1896). *Ber. dtsch. chem. Ges.* **29**, 2355.
Frieden, E. & Winzler, R. J. (1949). *J. biol. Chem.* **179**, 423.
Kalb, L., Schweizer, F., Zellner, H. & Barthold, E. (1926). *Ber. dtsch. chem. Ges.* **59**, 1869.
Maclagan, N. F. & Sheahan, M. M. (1950). *J. Endocrinol.* **6**, 456.
Maclagan, N. F., Sheahan, M. M. & Wilkinson, J. H. (1949). *Nature, Lond.*, **164**, 699.
Sheahan, M. M., Wilkinson, J. H. & Maclagan, N. F. (1951). *Biochem. J.* **48**, 188.
Wheeler, H. L. & Liddle, L. M. (1910). *Amer. chem. J.* **42**, 441.
Wilkinson, J. H. (1951). *J. chem. Soc.* p. 626.

The Biological Action of Substances Related to Thyroxine

3. SUBSTANCES DERIVED FROM 3:5-DIIODO-4-HYDROXYBENZALDEHYDE AND RELATED COMPOUNDS

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The dimethylacetal of 3:5-diiodoanisaldehyde (Wilkinson, 1949) was shown by Maclagan, Sheahan & Wilkinson (1949) to exhibit an antithyroxine action when tested in mice by the oxygen-consumption method (Maclagan & Sheahan, 1950). This effect was only produced at a total dosage of 1000 mg./kg. and it was the object of the work described in the present communication to survey a series of related compounds in the hope of finding more effective substances. Frieden & Winzler (1949) have shown that 3:5-diiodo-4-benzyloxybenzoic acid was active by the amphibian-metamorphosis test, a result we have confirmed in mice by the oxygen-consumption test (Wilkinson, Sheahan & Maclagan, 1951). Accordingly, we decided to examine the benzyl ether of 3:5-diiodo-4-hydroxybenzaldehyde and its dimethylacetal, but as frequently occurs when two supposedly active groups are combined in one compound, the products had no pharmacological action. We therefore turned our attention to a more systematic study of derivatives of a series of ethers of 3:5-diiodo-4-hydroxybenzaldehyde and related substances.

The ethyl and benzyl ethers of the hydroxy aldehyde were obtained by the methods used for the preparation of the corresponding ethers of the diiodohydroxybenzoates (Wilkinson *et al.* 1951). The ethyl ether was characterized as its oxime and dimethyl acetal, and the benzyl derivative was converted into its dimethyl acetal by the usual procedure. By similar means 3:5-diiodo-2-methoxybenzaldehyde and its dimethyl acetal were prepared from 3:5-diiodosalicylaldehyde. Some esters of 3:5-diiodo-4-hydroxybenzaldehyde were obtained by standard methods and some of these were readily converted into the corresponding acetals.

In order to determine whether mono-iodo substituted compounds were likely to be of any interest we prepared 5-iodovanillin and 5-iodoveratraldehyde. The latter was obtained by the methylation of the former by the method of Kalb, Schweizer, Zellner & Barthold (1926) using a large excess of methyl sulphate. Less drastic conditions failed to produce more than a trace of the required veratraldehyde.

EXPERIMENTAL

Chemical methods

Micro-analyses were by Drs Weiler and Strauss, Oxford. All melting points are uncorrected.

The following new derivatives of 3:5-diiodoanisaldehyde are reported:

Oxime. Colourless needles, m.p. 153°, from benzene. Highly soluble in most organic solvents, except light petroleum. (Found: C, 24·0; H, 1·7; I, 62·8. $C_8H_7O_2NI_2$ requires C, 23·8; H, 1·8; I, 63·1%.)

Diethyl acetal. Diiodoanisaldehyde (1 g.) was heated under reflux for 1 hr. with ethanol containing 1% (w/w) HCl (10 ml.) and, after cooling, the solution was treated with ice and Na_2CO_3. The solid (0·89 g.) was collected and dried over calcium chloride. The compound crystallized from light petroleum (b.p. 40–60°) in colourless rhombic prisms, m.p. 76°. (Found: C, 31·2; H, 3·5; I, 55·4. $C_{12}H_{16}O_3I_2$ requires C, 31·2; H, 3·5; I, 55·0%.)

3:5-*Diiodo-2-methoxybenzaldehyde.* 3:5-Diiodosalicylaldehyde (10 g.) was suspended in methanol (70 ml.) and treated with dimethyl sulphate (16 g.). 40% NaOH (16 ml.) was added and, after the vigorous reaction had subsided, the mixture was set aside overnight. Water (200 ml.) was added and the acetal which separated was extracted with ether, dried (Na_2SO_4) and freed from solvent. As it could not be induced to crystallize and decomposed when distillation at 0·1 mm. was attempted, the acetal was hydrolysed by boiling with 2N-HCl (10 ml.) for 15 min. The *aldehyde* (5·42 g.), which solidified on cooling, separated from light petroleum (b.p. 80–100°) in colourless needles, m.p. 100°. (Found: C, 24·8; H, 1·7; I, 65·4. $C_8H_6O_2I_2$ requires C, 24·7; H, 1·6; I, 65·5%.) The *dimethyl acetal* was obtained from the purified aldehyde (1 g.) by refluxing with methanol containing 1% (w/w) HCl (10 ml.) for 1 hr., after which the solution was treated with ice and Na_2CO_3. Extraction with ether furnished a yellow viscous oil (1·15 g.) which again failed to crystallize. (Found: C, 28·2; H, 2·9; I, 58·9. $C_{10}H_{12}O_3I_2$ requires C, 27·7; H, 2·8; I, 58·6%.)

3:5-Diiodo-4-ethoxybenzaldehyde, its oxime and dimethyl acetal were prepared as described by Wilkinson (1951).

3:5-*Diiodo-4-benzyloxybenzaldehyde.* 3:5-Diiodo-4-hydroxybenzaldehyde (7·5 g., 0·02 g.mol.) was suspended in ethanol (50 ml.) and treated with 2N-NaOH (30 ml., 0·06 g.mol.). Benzyl chloride (7·6 g., 0·06 mol.) was added and the mixture boiled under reflux for 1 hr., when it was no longer alkaline to litmus. An oil, which hardened to a gum, separated on cooling. Repeated crystallization from 90% ethanol gave the required *aldehyde* (4·0 g.) as colourless needles, m.p. 103°. (Found: C, 36·2; H, 2·2; I, 55·2. $C_{14}H_{10}O_2I_2$ requires C, 36·2; H, 2·2; I, 54·7%.) The 2:4-*dinitrophenylhydrazone* separated from dioxan in orange-red needles, m.p. 263–265° (decomp.). (Found: N, 8·8. $C_{20}H_{14}O_5N_4I_2$ requires N, 8·7%.) The *dimethyl acetal* was obtained by heating under reflux a mixture of the aldehyde (2 g.) and anhydrous $CaCl_2$ (0·3 g.) in methanol (6 ml.) for 2 hr. The oil, which separated on dilution with water, hardened overnight, and, after crystallization from methanol, formed colourless plates, m.p. 95°. (Found: C, 37·6; H, 3·1; I, 49·9. $C_{16}H_{16}O_3I_2$ requires C, 37·7; H, 3·1; I, 49·8%.)

3:5-*Dibromoanisaldehyde dimethyl acetal* was obtained by treating a solution of 3:5-dibromo-4-hydroxybenzaldehyde (28 g.) in methanol (150 ml.) and dimethyl sulphate (50 g.) with 40% NaOH (50 ml.) as described under 3:5-diiodo-2-methoxybenzaldehyde. On dilution with water the *acetal* (23·15 g.) separated as an oil which, on treatment with ether, hardened to yellow solid, m.p. 58–59°. Recrystallization from light petroleum (b.p. 40–60°) gave pale-yellow plates, m.p. 62–63°. (Found: C, 35·8; H, 3·4; Br, 48·6. $C_{10}H_{12}O_3Br_2$ requires C, 35·3; H, 3·5; Br, 47·1%.) Hydrolysis with 2N-HCl by boiling under reflux for 20 min. gave the *aldehyde* in 97% yield. It crystallized from ethanol in colourless needles, m.p. 85°. (Found: C, 33·4; H, 2·2; Br, 54·1. $C_8H_6O_2Br_2$ requires C, 32·6; H, 2·1; Br, 54·4%.)

3:5-*Diiodo-4-acyloxybenzaldehydes.* 3:5-Diiodo-4-hydroxybenzaldehyde (9·35 g., 0·025 mol.) was dissolved in pyridine (15 ml.) and treated with the appropriate acid anhydride or acid chloride (0·03 mol.). After 30 min. at room temperature, the solution was heated to 60° for 10 min., cooled and diluted with water (50 ml.). The esters were usually obtained as gums, which hardened on standing, though in some cases treatment with ethanol was necessary to induce crystallization. (Yields: 80–95%.) By this means the following were prepared:

Acetate. Colourless needles, m.p. 158°, from 90% ethanol. (Found: C, 26·4; H, 1·5; I, 61·2. $C_9H_6O_3I_2$ requires C, 26·0; H, 1·45; I, 61·1%.)

The *dimethyl acetal* formed colourless prisms, m.p. 102°, from methanol. (Found: C, 28·6; H, 2·6; I, 55·0. $C_{11}H_{12}O_4I_2$ requires C, 28·6; H, 2·6; I, 55·0%.)

Propionate. Pale-buff crystals, m.p. 127°, from light petroleum (b.p. 80–100°). (Found: C, 28·2; H, 1·8; I, 58·8. $C_{10}H_8O_3I_2$ requires C, 27·9; H, 1·9; I, 59·1%.)

The *dimethyl acetal* separated from light petroleum (b.p. 40–60°) in colourless prisms, m.p. 54–55°. (Found: C, 29·8; H, 2·9; I, 53·6. $C_{12}H_{14}O_4I_2$ requires C, 30·3; H, 2·95; I, 53·4%.)

n-*Butyrate.* Pale-buff crystals, m.p. 81–82°, from light petroleum (b.p. 60–80°). (Found: C, 29·9; H, 2·1; I, 57·0. $C_{11}H_{10}O_3I_2$ requires C, 29·7; H, 2·3; I, 57·2%.)

Benzoate. Buff needles from ethanol, m.p. 138–140°. (Found: C, 35·1; H, 1·8; I, 52·8. $C_{14}H_8O_3I_2$ requires C, 35·2; H, 1·7; I, 53·1%.)

Phenylacetate. Buff crystals from ethanol, m.p. 133–134°. (Found: C, 36·8; H, 2·1; I, 51·5. $C_{15}H_{10}O_3I_2$ requires C, 36·6; H, 2·0; I, 51·6%.)

Toluene-p-sulphonate. Colourless prisms from 70% ethanol, m.p. 133°. (Found: C, 31·9; H, 2·1; I, 47·8. $C_{14}H_{10}O_4I_2S$ requires C, 31·8; H, 1·9; I, 48·1%.) The *dimethyl acetal* separated from benzene in colourless crystals, m.p. 198–199°. (Found: C, 34·7, 34·9; H, 3·0, 2·9; I, 43·8; S, 5·8. $C_{16}H_{16}O_5I_2S$ requires C, 33·5; H, 2·8; I, 44·2; S, 5·6%.)

3:5-*Diiodo-4-hydroxypropiophenone.* 4-Hydroxypropiophenone (1 g.) was suspended in NH_3 solution (sp.gr. 0·88; 10 ml.) and treated over 1·5 hr. with a solution of I_2 (3·4 g.) and KI (4 g.) in water (20 ml.). The mixture was set aside overnight, acidified with HCl, and filtered. The solid (2·54 g., 94%) crystallized from ethanol in colourless needles, m.p. 122°. (Found: C, 26·9; H, 1·8; I, 63·4. $C_9H_8O_2I_2$ requires C, 26·9; H, 2·0; I, 63·2%.) The *acetyl* derivative separated from ethanol in colourless highly refractive needles, m.p. 155°. (Found: I, 56·7. $C_{11}H_{10}O_3I_2$ requires I, 57·2%.)

3:5-*Diiodo-4-methoxypropiophenone.* The 4-hydroxy compound (4 g.) was suspended in a mixture of methanol (20 ml.) and methyl sulphate (3·8 g.) and treated with 10N-NaOH (3 ml.). The *product* (3·36 g., 81%) was isolated on dilution with water (50 ml.). It crystallized from light petroleum (b.p. 80–100°) in colourless rhombic plates, m.p. 83–84°. (Found: C, 28·6; H, 2·6; I, 61·1. $C_{10}H_{10}O_2I_2$ requires C, 28·8; H, 2·4; I, 61·1%.)

5-*Iodoveratraldehyde.* Iodovanillin (13·9 g.) was dissolved in methyl sulphate (50 ml.) at 100°. 20% NaOH was added gradually with vigorous shaking until the solution was alkaline to phenolphthalein. About 45 min. was required for the addition. The gum which separated crystallized on cooling. It was collected, washed with water and dried over silica gel at 20°. Yield: 13·4 g. (92%). Crystallization from ethanol gave pure 5-*iodoveratraldehyde* in colourless needles, m.p. 69–70°. (Found: C, 37·4; H, 3·2; I, 43·1. $C_9H_9O_3I$

requires C, 37·0; H, 3·1; I, 44·5%.) The *oxime* separated from ethanol in colourless prisms, m.p. 110–111°. (Found: N, 4·4; I, 41·3. $C_9H_{10}O_3NI$ requires N, 4·6; I, 41·3%.)

The 3:5-diiodo-4-hydroxybenzaldoxime was prepared as described by Paal & Mohr (1896).

Biological methods

The compounds were tested in thyroxine-treated mice by the oxygen-consumption test (Maclagan & Sheahan, 1950) with the improvements described by Sheahan, Wilkinson & Maclagan (1951).

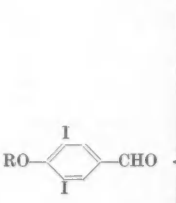
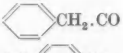
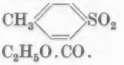
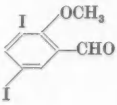
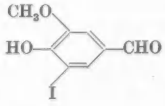
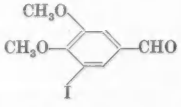
RESULTS

The results obtained with a series of ethers and esters of 3:5-diiodo-4-hydroxybenzaldehyde are given in Table 1. With the exception of the carbethoxy derivative and the acetate, which caused significant reductions in the thyroxine effects at total dosages of 200 and 400 mg./kg. respectively, compounds containing the free aldehyde group had little anti-thyroxine activity. In very high dosage (1000 mg./kg.) the ethyl ether, however, did produce an effect.

The series of acetals also failed to produce any highly active compounds. Apart from the dimethyl acetal of 3:5-diiodoanisaldehyde which, as has already been reported, was active at 1000 mg./kg., the dimethyl acetal of 3:5-diiodo-4-ethoxybenzaldehyde was the only compound which caused any significant diminution in the response to thyroxine (Table 2).

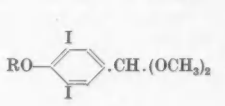
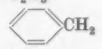
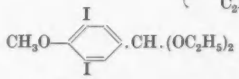
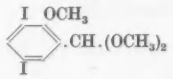
By far the most active members of this series of iodinated aldehydes were found amongst the oximes (Table 3). 3:5-Diiodoanisaldoxime, which was active at a total dose of 50 mg./kg., had a potency only slightly less than that of *n*-butyl 3:5-diiodo-4-hydroxybenzoate, the most active compound we have so far examined. The corresponding ethoxy derivative was also active, though at doses below 400 mg./kg. it was ineffective.

Table 1. *The antithyroxine activity of ethers and esters of* 3:5-*Diiodo*-4-*hydroxybenzaldehyde and related compounds*

Compound	Total dosage (mg./kg.) which produced: Significant response*	No significant response
 R = H	—	400
R = CH_3	—	1000
R = C_2H_5	1000 (74%)	400
R =	—	400
R = $CH_3.CO$	400 (67%)	200
R = $C_2H_5.CO$	—	400
R = n-$C_3H_7.CO$	—	400
R =	—	400
R = 	—	400
R = 	—	400
R = $C_2H_5O.CO.$	200 (24%)	100
	—	400
	—	400
	—	400

* The figures in brackets indicate the percentage inhibition of the thyroxine effect at the stated dose. The figures were subjected to statistical analysis by 'Student's' *t* test.

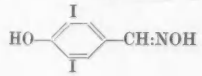
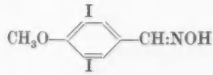
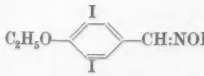
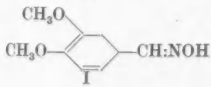
Table 2. *The thyroxine-inhibitory activity of ethers and esters of 3:5-diiodo-4-hydroxybenzaldehyde dialkyl acetals and similar compounds*

Compound		Total dosage (mg./kg.) which produced: Significant response*	Total dosage (mg./kg.) which produced: No significant response
	R = CH_3	1000 (82%)	400
	C_2H_5	1000 (36%)	400
		—	1000
	$CH_3.CO$	—	400
	$C_2H_5.CO$	—	400
		—	1000
		—	400

* See footnote to Table 1.

5-Iodovanillin, 5-iodoveratraldehyde and 5-iodoveratraldoxime were tested as examples of mono-iodo derivatives but none was active at 400 mg./kg. 3:5-Dibromoanisaldehyde and its dimethyl acetal were examined, but were also inactive at this dosage.

Table 3. *The antithyroxine activity of oximes of 3:5-diiodo-4-hydroxybenzaldehyde and related compounds*

Compound	Total dosage (mg./kg.) which produced: Significant response*	Total dosage (mg./kg.) which produced: No significant response
	—	400
	50 (57%)	25
	400 (68%)	200
	—	400

* See footnote to Table 1.

Two ketones, 4-hydroxy- and 4-methoxy-3:5-diiodopropiophenones, were also tested, but at high dosage they proved to be toxic. The maximum tolerated dose of the former was 50 mg./kg., at which it was without effect on the oxygen consumption. The methoxy derivative was rather less toxic and at a total dose of 100 mg./kg. it produced a 36% inhibition of the thyroxine effect.

DISCUSSION

It is noteworthy that the carbethoxy derivative of 3:5-diiodo-4-hydroxybenzaldehyde was the most active ester of this aldehyde, since, in the preceding paper, it has been shown that, whilst esterification of the phenolic hydroxyl group of *n*-butyl 3:5-diiodo-4-hydroxybenzoate reduced the antithyroxine activity, the carbethoxy derivative exhibited the smallest diminution. In general, however, the aldehydes were much less effective than those series derived from 3:5-diiodo-4-hydroxybenzoic acid.

The high potency of 3:5-diiodoanisaldoxime is in marked contrast to the weak activity of the other derivatives, but in our survey of aldehyde derivatives, it appears that this compound represents a peak since 3:5-diiodo-4-hydroxybenzaldoxime was inactive and the corresponding 4-ethoxy compound showed only a moderate thyroxine-inhibitory action.

SUMMARY

1. A total of twenty-four compounds chemically related to 3:5-diiodo-4-hydroxybenzaldehyde have been examined for antithyroxine activity by the oxygen-consumption method in mice.

2. Of these, 3:5-diiodo-4-ethoxybenzaldehyde and its dimethyl acetal and 3:5-diiodoanisaldehyde dimethyl acetal showed weak activity; the acetyl and carbethoxy derivatives of 3:5-diiodo-4-hydroxybenzaldehyde and 3:5-diiodo-4-ethoxybenzaldoxime were moderately active, whilst 3:5-diiodoanisaldoxime was highly active.

3. Certain mono-iodo compounds related to 5-iodovanillin were also tested, but were without action.

The authors have much pleasure in acknowledging the continued support given to the research reported in this and the preceding paper by Glaxo Laboratories Ltd.

REFERENCES

Frieden, E. & Winzler, R. J. (1949). *J. biol. Chem.* **179**, 423.
Kalb, L., Schweizer, F., Zellner, H. & Barthold, E. (1926). *Ber. dtsch. chem. Ges.* **59**, 1869.
Maclagan, N. F. & Sheahan, M. M. (1950). *J. Endocrinol.* **6**, 456.
Maclagan, N. F., Sheahan, M. M. & Wilkinson, J. H. (1949). *Nature, Lond.*, **164**, 699.
Paal, C. & Mohr, L. (1896). *Ber. dtsch. chem. Ges.* **29**, 2302.
Sheahan, M. M., Wilkinson, J. H. & Maclagan, N. F. (1951). *Biochem. J.* **48**, 188.
Wilkinson, J. H. (1949). *J. chem. Soc.* p. 2370.
Wilkinson, J. H. (1951). *J. chem. Soc.* (in the Press).
Wilkinson, J. H., Sheahan, M. M. & Maclagan, N. F. (1951). *Biochem. J.* **49**, 710.

Comparative Studies of 'Bile Salts'

3. THE CONVERSION OF PYTHOCHOLIC ACID TO DEOXYCHOLIC ACID

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Guy's Hospital Medical School, London, S.E. 1

(*Received* 11 *May* 1951)

Pythocholic acid, $C_{24}H_{40}O_5$, from the bile of Boidae was shown, as reported in Part 2 of this series (Haslewood & Wootton, 1951), to be a cholanic acid having three secondary hydroxyl groups substituted probably at C-3, C-12 and C-15 or C-16 in the molecule. Such a substance might be convertible to ordinary deoxycholic acid (3α:12α-dihydroxycholanic acid), and the present report gives details of successful experiments leading to this end. Opportunity is taken to record an investigation of the bile salts of two more species of Boidae.

RESULTS AND DISCUSSION

The dibenzoate (I) of pythocholic lactone was readily obtained. When this was dissolved in warm dilute ethanolic potassium hydroxide, the lactone ring was opened. The resulting acid was precipitated at once and converted into its methyl ester. Without purification, this was oxidized with chromic oxide and a non-crystalline product, which must have consisted chiefly of (II), was isolated. Treatment of this by the Kishner-Wolff method gave deoxycholic acid (III) in a yield of 71%, calculated on the crystalline dibenzoate. Since cholic acid can be converted to deoxycholic acid without change of configuration and in good yield by essentially similar methods (Gallagher & Long, 1943; Haslewood, 1943; Schneider & Hoehn, 1943), the above experiments leave no reasonable doubt that pythocholic lactone is correctly formulated as a 3α:12α-dihydroxy compound; pythocholic acid is therefore almost certainly 3α:12α:16(or 15)-trihydroxycholanic acid.

Results of an examination of the bile salts of two more species of Boidae are given in Table 1. These figures continue to support the view that pythocholic acid is characteristic of the bile of Boidae.

Table 1. *Approximate amounts of bile acids in two species of Boidae*

(Details as described by Haslewood & Wootton, 1951.)

Species	Pythocholic acid (% of 'bile acids'; approx. minimal figures)	Cholic acid (% of 'bile acids'; approx. minimal figures)
Anaconda, *Eunicotes murinus*	25	8
Reticulated python, *Python reticulatus*	48	8

EXPERIMENTAL

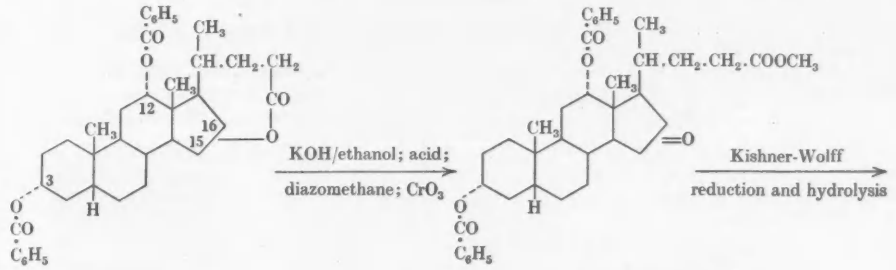
General. All melting points are uncorrected. Al_2O_3 was obtained from Hopkin and Williams and neutralized as described by Shoppee (1949). Micro-analysis was done by Drs Weiler and Strauss, Oxford. Optical rotations were determined in a 1 dm. micro tube. 20% CrO_3=a solution made by dissolving CrO_3 (20 g.) in the minimal amount of water and making to 100 ml. with acetic acid.

Conversion of pythocholic lactone to deoxycholic acid

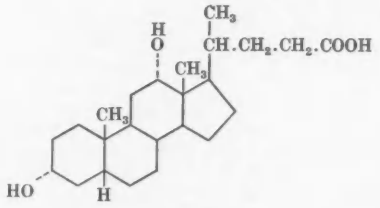
Pythocholic lactone dibenzoate. The lactone (50 mg.) was dissolved in pyridine (0·5 ml.) with benzoyl chloride (0·2 ml.) and the solution heated at about 90° for 30 min. It was cooled and diluted with excess aqueous HCl. Methanol was now added to a concentration of about 60% (v/v) and the solid which gradually formed was collected, washed with approx. 50% (v/v) methanol-water and crystallized from ethanol. Yield, 45 mg., m.p. 198–202°. This substance was

recrystallized from ethanol, from which it appeared as short white needles.

Pythocholic lactone dibenzoate had m.p. 200–201°. (Found: C, 76·1; H, 8·0. $C_{38}H_{46}O_6$ requires C, 76·3; H, 7·7 %.) The substance was recovered unchanged (m.p. 199–201°, not depressed by the starting material) after treatment in the cold with 20 % CrO_3 in acetic acid.



(I) (II) not crystallized



(III)

Conversion of the dibenzoate to a product containing (II). The dibenzoate (0·33 g.) was dissolved in ethanol (33 ml.) and treated at about 60° with KOH (3·3 ml. of an approx. 40 % (w/v) aqueous solution). As soon as the alkali had been added, the flask was shaken and the mixture at once diluted with 2–3 vol. of cold water. The solution, which had remained clear, was acidified immediately with aqueous HCl. The flocculent white precipitate was collected as quickly as possible on a filter, washed with water and dissolved in ethanol. This was saturated with diazomethane, carried over in N_2 from decomposing *N*-nitrosomethylurea (1 g.). The solution was evaporated on the water pump. The gummy residue, which showed no tendency to crystallize with organic solvents, was dissolved in acetic acid (4 ml.) at 25° and treated with 20 % CrO_3 (0·4 ml.). After 15 min. at 25° with occasional shaking, the solution was diluted with water, and when separation of a solid seemed complete, this was collected and washed with water. This product showed no tendency to crystallize. It was dried by evaporation with ethanol, dissolved in benzene and the solution run on to Al_2O_3 (3·5 g.) in a column. Benzene (140 ml.) eluted the whole of the material (0·33 g.).

Deoxycholic acid from (II). The above material (0·33 g.) was transferred with ether to a small metal bomb. After evaporation of the ether, the residue was dissolved in ethanol (5 ml.) in which was dissolved sodium (0·3 g.) and hydrazine hydrate (0·5 ml. of approx. 95 %). The bomb was sealed and heated at 200–210° for 4 hr. When the cooled bomb was opened it was found that the solvent had evaporated. The white residue was dissolved in water and the solution acidified with HCl. The precipitated acid was collected, washed, dissolved in ethanol and the solution filtered. Evaporation left a residue which with ether gave the deoxycholic acid-ether complex as white needles of m.p. 155–160°. Yield 0·155 g. (71 %, calculated on the weight of dibenzoate used). From acetic acid, this substance gave the deoxycholic-acetic acid complex as typical white needles, m.p. 139–141°, not depressed by authentic material prepared from ox-bile. $[\alpha]_D^{19°} = +45° \pm 1°$ in ethanol (*c*, 1·0). The product (50 mg.) of m.p. 139–141° was oxidized in the usual way with CrO_3 to give dehydrodeoxycholic acid, crystallizing from dilute ethanol as typical glistening leaflets of m.p. 177–179°, not depressed by authentic material. $[\alpha]_D^{19°} = 87° \pm 2°$ in ethanol (*c*, 0·7).

Methyl pythocholate, pythocholic lactone and cholic acid from Eunicotes murinus *and* Python reticulatus

These were prepared exactly as described in Part 2 (Haslewood & Wootton, 1951) for other species of Boidae. No unexpected difficulties were encountered.

SUMMARY

1. Pythocholic lactone dibenzoate was prepared. When this was dissolved in ethanolic alkali, the lactone ring was opened without significant hydrolysis of benzoyl groups. Re-lactonization was prevented by methylation of the carboxyl group, and the free hydroxyl group was then oxidized to

carbonyl by chromium trioxide. Kishner-Wolff reduction of the non-crystalline product gave deoxycholic acid in good yield. Pythocholic acid is therefore almost certainly 3α:12α:16(or 15)-trihydroxycholanic acid.

2. The bile salts of two more species of Boidae have been examined. Both contained pythocholic acid and smaller amounts of cholic acid.

I gratefully acknowledge the gift of Boa bile by Dr O. Hill, Mr W. E. Lawrence and the Zoological Society of London.

REFERENCES

Gallagher, T. F. & Long, W. P. (1943). *J. biol. Chem.* **147**, 131.
Haslewood, G. A. D. (1943). *Biochem. J.* **37**, 109.
Haslewood, G. A. D. & Wootton, V. M. (1951). *Biochem. J.* **49**, 67.
Schneider, A. W. & Hoehn, W. M. (1943). *J. Amer. chem. Soc.* **65**, 485.
Shoppee, C. W. (1949). *J. chem. Soc.* p. 1671.

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